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UNIVERSITY OF ALBERTA

# SIGNIFICANCE OF TH2-TYPE CYTOKINES AT THE MATERNAL-FETAL INTERFACE

by

HUI LIN ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (IMMUNOLOGY)

Edmonton, Alberta
Fall, 1993

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FACULTY OF GRADUATE STUDIES AND RESEACH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SIGNIFICANCE OF TH2 TYPE CYTOKINES AT THE MATERNAL-FETAL INTERFACE submitted by HUI LIN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MEDICAL SCIENCES (IMMUNOLOGY).

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Date: Aug 18/93

Dedicated to my parents and my wife Wang Qi,
for their love and encouragement.

# ABSTRACT

The maternal immune response appears biased towards antibody production and away from cell-mediated immunity during pregnancy. Since the cellular immunity and antibody responses are regulated by TH1 and TH2 cytokines, these cytokines might be produced and involved in the regulation of the maternal immune response. We examined the local and distal release of cytokines during murine pregnancy using ELISAs. We found that the TH2-type cytokines IL-4, IL5, and IL10, along with IL-3 were readily detectable in cell supernatants derived from fetal-placental units in all three trimesters of gestation. These cytokines were detected in lysates of freshly isolated day 12 decidual and placental cells and in supernatants as early as 15 minutes after the begining of culture. IFN-γ was also found at early stages of pregnancy. Compared with the cytokine profile of Con A-stimulated normal spleen, cytokine production of the interface tissues was significantly biased towards a TH2 cytokine profile. None of these cytokines were produced by mesenteric lymph node or para-aortic lymph node cells from pregnant mice. Spleen cells from pregnant mice secreted IFN-γ only with stimulation of Con A.

IL-3, IL-4, and IL-10, but not IL-5 and IFN-γ were produced by cultured interface tissues from SCID and SCID·beige mice prepared in Dr. Croy's laboratory at the University of Guelph. IL-10-producing cells were further localized to the maternal decidua at day 6 of gestaion by *In situ* hybridization. The production of IL-3, IL-4, and IL-10 by cultured interface tissues, prepared in Dr. Chaouat's

laboratory in Clamart, France, was reduced in fetal resorption-prone CBA X DBA/2 mice. The production of these cytokines returned to normal levels when the fetal resorption of CBA X DBA/2 mice was prevented after ovine trophoblast protein was injected into these mice in Dr. Chaouat's laboratory. These observations indicate that TH2-type cytokines are normally produced at the maternal-fetal interface. The production of these cytokines may be involved in maintaining a successful pregnancy, and they may also influence the maternal systemic immune response.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AFP: | α-Fetoprotein |
| AIDS: | Aquired Immunodeficiency Syndrom |
| B Cell: | Bone Marrow Derived Lymphocyte |
| bp: | Base Pair |
| CD: | Cluster of Differentiation |
| CMI: | Cell-mediated Immunity |
| Con A: | Concanavalin A |
| CSF: | Colony Stimulating Factor |
| CTL: | Cytotoxic T Lymphocyte |
| dsRNA: | Double Stranded RNA |
| DTH: | Delayed Type Hypersensitivity |
| ELISA: | Enzyme-linked Immunosorbent Assay |
| FCS: | Fetal Calf Serum |
| FPLC: | Fast Protein Liquid Chromatography |
| GM-CSF: | Granulocyte-macrophage Stimulating Factor |
| HCG: | Human Chorionic Gonadotropin |
| HIV: | Human Immunodeficiency Virus |
| HLA: | Human Leukocyte Antigen |
| HPL: | Human Placental Lactogen |
| IFN-γ: | Interferon-γ |
| Ig: | Immunoglobulin |
| IL: | Interleukin |
| kd: | Kilodalton |
| LAK: | Lymphokine-activated Killer Cell |
| LIF: | Leukemia Inhibitory Factor |

| | |
|---|---|
| LT: | lymphotoxin |
| MHC: | Major Histocompatibility Complex |
| MLN: | Mesenteric Lymph Node |
| NK: | Natural Killer |
| oTP: | Ovine Trophoblast Protein |
| PALN: | Para-aortic Lymph Node |
| PAG: | Pregnancy Associated α2-Glycoprotein |
| PBS: | Phosphate Buffered Saline |
| PHA: | Phytohaemagglutinin |
| PIBF: | Progesterone-induced Blocking Factor |
| PMA: | Phorbol Myristate Acetate |
| RA: | Rheumatoid Arthritis |
| RT: | Room Temperature |
| SCID: | Severe Combined Immunodeficiency |
| SLE: | Systemic Lupus Erythematosus |
| T Cell: | Thymus-derived Lymphocyte |
| TGF-β: | Transforming Growth Factor-β |
| TH: | T Helper |
| TNF-α: | Tumor Necrosis Factor-α |

# I. INTRODUCTION

## A. Developmental and Functional Anatomy of Mouse Placenta.

The placenta serves as a functional connection between the developing embryo and its mother. It allows the biological exchange of nutrients, as well as respiratory and excretory substances. The development of the embryonic and maternal components, namely the trophoblast and endometrial decidua, which make up the major sites of the maternal-fetal interface, follows a well-defined pattern.

### 1. Placenta.

After fertilization, the ovum undergoes cleavage divisions and forms a solid sphere of cells called the morula. By 3 - 3.5 days post conception, the morula acquires an eccentrically placed cavity filled with fluid. The embryo at this stage is called the blastocyst. The outer layer of the blastocyst, the trophectoderm, is thickened at one point where a collection of cells, known as the inner cell mass, bulges into the blastocyst cavity. The trophectoderm eventually gives rise to membranes surrounding the embryo, while the inner cell mass forms the embryo proper.

The uterus of the non-pregnant mouse consists of three layers: the outer serosa, the myometrium and the endometrium. The endometrium consists of the lamina propria, the lining epithelium, the uterine glands and many blood vessels. Implantation involves penetration of the blastocyst through the uterine epithelium. The process starts around day 4.5, and on about day 6 the embryo is totally submerged in the endometrium from which it receives protection and nourishment during pregnancy.

During implantation the trophectoderm overlying the inner cell mass at the embryonic pole of the blastocyst undergoes rapid proliferation to form the ectoplacental cone, which is comprised of highly invasive trophoblast cells. The ectoplacental cone soon differentiates into three types of chorionic trophoblasts: (1) trophoblastic giant cells, (2) labyrinthine trophoblast, and (3) spongiotrophoblast.

The giant cells are actively phagocytic and invade maternal tissues until contact is made with maternal blood vessels. Labyrinthine trophoblast cells form densely packed cell strands and clefts which merge into labyrinthine tubules and eventually fuse into the arteries and veins of the umbilicus. Spongiotrophoblast cells, which are situated at poorly vascularized areas of cells and extracellular matrix between labyrinthine trophoblast and endometrium, invade into the endometrium. The invasive trophoblasts continue to erode the maternal stroma until they penetrate the small capillaries which then supply the trophoblast cells with an external source of nutrients.

## 2. Decidua.

Certain undetermined signals, which are related to hormones secreted by trophoblasts and their presence as a stimulant, cause the endometrial cells to enlarge and proliferate. This procedure is called the decidual reaction, the differentiated endometrial stromal cells being called decidual cells. The decidua can be divided into the decidua basalis, the decidua capsularis, and the decidua parietalis.

Decidual tissue is heterogenous in composition, comprising typical stromal type cells, hematogenous leukocytes, metrial gland cells and other stromal elements. The decidual stromal cells are the most frequent cell class in the decidual tissue (Kearns and Lala, 1985), most of them being bone marrow derived (Kearns and Lala, 1982). Leukocytes are comprised of lymphocytes, monocyte-macrophages and granulocytes. Lymphocytes, the second overall most frequent cell class, are made up of T cells (25 - 45%), B cells (10 -13%), and null cells (45 - 80%). Macrophages, which express Mac-1, are the third most commonly occuring cells at the decidua (Kearns and Lala, 1985). The granulocytes are the least common leukocytes of the three. The granulated metrial gland (GMG) cell is derived from lymphoid-like cells during early pregnancy and differentiates into large granulated cells at a later stage (Peel, 1989). GMG cells represent 15-20% of the cells in metrial glands (Stewart and Peel, 1978; Stewart and Peel, 1982). The stromal elements include fibroblast cells and blood vessels.

### 3. Major Histocompatibility Antigen Expression on Trophoblast

Late morulas and early blastocysts express serologically detectable Class I major histocompatibility (MHC) antigens (Warner and Spannaus, 1984; Goldbard et al, 1985). However, MHC antigens are not detectable on the outer trophectoderm by the late blastocyst stage (Lala et al, 1984). Therefore, just prior to implantation, trophectodermal Class I MHC antigens are lost or masked.

By day 9 of gestation, trophoblast cells re-express Class I MHC antigens at low levels. *In situ* labeling of placenta, by injection of radiolabeled anti-paternal MHC antibodies into the uterine artery, has revealed that the spongiotrophoblast is weakly Class I MHC antigen positive (Singh et al, 1983). Strong specific immunohistochemical staining with anti-Class I antibodies was detected on spongiotrophoblast in cryostat sections of mature rat placenta (Billington and Burrows, 1986; Ho et al, 1987). In labyrinthine trophoblast, only a low level of Class I transcripts is detected by *in situ* hybridization (Philpott et al, 1988). Class I antigens are not detected at the protein level (Billington and Burrows, 1986).

The distribution of Class I MHC antigens within the human placenta is different from that of the mouse. The human extravillous trophoblast expresses both mRNA and protein of Class I MHC molecules (Sunderland et al, 1981; Redman et al, 1984; Hsi et al, 1984; Wells et al, 1984; Loke and Butterworth, 1987). Villous cytotrophoblast expresses only mRNA but no protein (Hunt and Orr, 1990), while neither mRNA nor protein is detected in villous syncytiotrophoblast (Faulk and Temple, 1976; Sunderland et al, 1981; Hunt and Orr, 1990).

The Class I MHC antigen expressed on human trophoblast is a product of the non-classical Class I gene that codes for human leukocyte antigen-G (HLA-G) (Ellis et al, 1990). Analysis of HLA-G expression shows that it is not polymorphic and that the 37 kd acidic isoform is secreted (Hunt and Orr, 1992; Wei and Orr, 1992; Kovats et al, 1990).

In contrast to Class I MHC antigens, Class II MHC antigens have not been detected on trophoblast at any stage of gestation in the mice (Jenkinson and Searle, 1979; Raghupathy et al, 1981) or in humans (Goodfellow et al, 1976; Faulk and Temple, 1976; Sunderland et al, 1981).

## B. Effects of the Maternal Immune Response on the Development of Feto-placental Unit

### 1. Recognition of the Fetus by the Maternal Immune System

The maternal immune system recognizesre the presence of histo-incompatible fetus during normal pregnancy. In humans, the production of maternal antibodies against fetal (paternal) HLA antigens is relatively common and normal. Approximately 20% of primiparous and 50% of multiparous women produce alloantibodies reactive with paternal Class I HLA antigens (Van der Werf, 1971; Winchester et al, 1975) and Class II HLA antigens (Winchester et al, 1975). Since classical Class I and Class II HLA antigens are not expressed by trophoblasts, these antibodies may be induced by the exposure of other fetal tissues to the maternal immune system. The fact that alloantibodies can develop even during a first pregnancy (Van der Werf, 1971) suggests that immunization is not the result of events at delivery, in contrast to Rh isoimmunization.

In animals, alloantibodies are found in pregnant mice (Bell and Billington, 1980; Bell and Billington, 1981) and rats (Smith et al, 1982; Ghani et al, 1984). The alloantibodies are found to be against paternal MHC antigens as well as non-MHC antigens (Asfar et al, 1985; Power et al, 1987).

Maternal recognition of the fetus is also reflected by an influx of lymphocytes into the decidua (Lala et al, 1986). This occurs shortly after the expression of fetal Class I MHC antigens on the surface of invasive trophoblasts (Raghupathy et al, 1981). It has also been reported that the lymph nodes which drain the uterus in outbred rodent pregnancies are larger than those in inbred pregnancies (Head et al, 1981).

## 2. Influence of the Maternal Immune System on Fetal Growth and Function

Although the effects of alloantibodies are not clear, there has been circumstantial evidence which suggests that a positive maternal immune response in pregnancy is important to the survival of the fetus. In the early 1980s, two different groups carried out clinical trials, using either women husbands' or pooled third party lymphocytes to treat patients who suffered from chronic spontaneous abortion of unknown etiology (Beer et al, 1985; Mowbray et al, 1987). The alloimmunization did lead to the birth of viable offspring. In the paired sequential trial done by Mowbray, alloimmunization produces a success rate of 78% in a subsequent pregnancy, much higher than in the placebo group (35%). However, more double-blind clinical trials need to be done to confirm this result.

In the meantime, progress was made in animal research. In 1980, Clark and his colleagues developed an animal model of spontaneous abortion (Clark et al, 1980). CBA female mice impregnanted by DBA/2 males were found to have an unusually high fetal resorption rate when examined at mid-gestation. The fetal resorption rate was between 30 to 50 percent, depending on the age of females and other factors. In contrast, other mouse strain combinations, including CBA X CBA, CBA X BALB/c, DBA/2 X DBA/2, DBA/2 X C3H, have resorption rates of 10 percent or less (Clark et al, 1980 and 1986).

In 1983, Wegmann and his colleagues found that the high fetal resorption rates of CBA X DBA/2 mice returned to normal after immunizing the females with white cells sharing an MHC haplotype with paternal strain cells (BALB/c) 7 days prior to conception. Surprisingly, the cells from paternal DBA/2 themselves had no effect for unknown reasons. But BALB/c cells, which share the MHC $H\text{-}2^d$ haplotype with paternal cells, reduced the fetal resorption rate from more than 90% to around 10% (Chaouat et al, 1983). Immunogenetic studies indicate that the H-2 haplotype of the BALB/c cells is necessary because BALB/c congenic cells lacking that haplotype do not provide protection against abortion (Kiger et al, 1985).

These results imply that maternal immunity has an influence on reproductive outcome. More evidence comes from studies of the role of maternal T cells during pregnancy. MRL-1pr/1pr strain mice have a T cell proliferative disorder. These mice also have unusually large placentas with abnormally high levels of phagocytosis (Chaouat et al, 1983). The placental size and phagocytosis can be reduced to normal by injecting anti-CD4 plus anti-CD8 monoclonal antibodies on day 8, 10 and 12 of gestation (Athanassakis et al, 1990).

Similar experiments have also been done with normal mice and T-cell deficient nude mice. In normal mice, anti-T cell antibody treatment reduces placental size and causes fetal resorption in the strain combinations CBA X BALB/c and CBA X CBA. Nude mice have small placentas, and treatment with anti-T cell antibody has no further effect on placental size and fetal resortion rate (Athanassakis et al, 1990). These experiments clearly indicate that maternal T cells regulate, directly or indirectly, the growth of the placenta, although successful breeding of nude and severe combined immunodeficiency (SCID) mice suggests that T cell immunity is not absolutely essential for successful pregnancy (Croy and Chapeau, 1990).

## C. Regulatory Effects of Cytokines on Fetal Growth and Function.

### 1. Enhancing Effects of some Cytokines on the Function of the Placenta and Fetus

Since T cells mediate many of their effects via secretion of cytokines, Wegmann and his colleagues postulated what is known as placental immunotrophism (Wegmann, 1984). The hypothesis states that maternal T cell recognition of the fetal allograft leads to release of cytokines that can serve as growth factors for trophoblasts and other placental cells, leading to improved placental function and fetal survival.

Based on this hypothesis, they carried out a series of experiments studying the relationship between cytokines of the colony stimulating factor (CSF) family and placental trophoblasts.

They found that phorbol myristate acetate (PMA)-treated EL4 lymphoma cell supernatants, as well as recombinant interlukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF) and colony stimulating factor-1 (CSF-1), stimulated mouse placental cell growth (Armstrong and Chaouat, 1989). Long term placental cell lines were also developed, whose growth was dependant on these cytokines (Athanassakis et al, 1987).

The cell type of the responsive cells that grew out of the placenta under these conditions was unknown. They were fetally derived and alkaline phosphatase positive (Athanassakis et al, 1987). They also were cytokeratin-positive and vimentin-negative, which is characteristic of epithelial but not macrophage cells (Athanassakis et al, 1987). However, they were Mac-1 and F4-80 positive, which is characteristic of macrophages (Athanassakis et al, 1987). In addition, CSF family cytokines also stimulated the phagocytic capacity of freshly isolated placental cells (Athanassakis et al, 1987).

When IL-3 and GM-CSF were injected into fetal resorption-prone CBA X DBA/2 mice, they reduced fetal resorption rates from 50% to 22% (IL-3) and from 47% to 8% (GM-CSF) (Chaouat et al, 1990). They also increased fetal and placental weights and expanded the spongiotrophoblast zone in the placenta (Chaouat et al, 1990).

Using *in situ* hybridization, Arceci and his colleagues found that a high level of CSF-1 receptor (CSF-1R) mRNA was expressed in the mouse trophoblast (Arceci et al, 1989), which provides the molecular basis for the effects of CSF-1 on placental cells. They also showed that the kinetics of CSF-1 synthesis correlate with the period of maximal placental growth (Arceci et al, 1989).

More recently, Robertson and her colleagues reported that GM-CSF increased embryonic implantation into uterine epithelial cells in culture ( Robertson and Seamark, 1991). When GM-CSF was added to cultures of purified human trophoblasts, it led to increased syncytialization of trophoblast cells and release of fetal growth hormone human placental lactogen (HPL) and human chorionic gonadotropin (HCG) (Guilbert et al, 1991). In addition, both GM-CSF and CSF-1 are secreted by human choriocarcinoma cell lines *in vitro*, and their proliferation can be blocked by adding antibodies to either GM-CSF or CSF-1 receptor to the culture (Garcia-Lloret et al, 1991).

## 2. Damaging Effects of some Cytokines on the Placenta and Fetus.

In addition to CSF cytokines, the effects of a variety of other cytokines have also been studied. A number of cytokines can compromise fetal and placental growth. Injection of tumor necrosis factor (TNF-α), interferon-γ (IFN-γ) and interleukin-2 (IL-2) increases fetal resorption rates in both abortion-prone CBA X DBA/2 mice and normal mice, including CBA X BALB/c and C3H X DBA/2 mice (Chaouat et al, 1990). TNF-α inhibits DNA synthesis of rat trophoblast cell-lines (Hunt and Orr, 1989) as well as embryonic development (Hill, et al, 1987).

These results expand the placental immunotrophism hypothesis to yield the concept that there are beneficial cytokines which can enhance fetal growth and survival and deleterious cytokines which can compromise pregnancy and lead to the death of the fetal-placental unit.

How do these deleterious cytokines compromise fetal growth? Although the exact pathway is not known, one of the mechanisms may be through the induction of natural killer (NK) activity.

The trophoblast is known to be relatively resistant to transplant rejection (King et al, 1989). It is also resistant to cytotoxic T lymphocyte (CTL) killing *in vitro* even when Class I alloantigens are upregulated by IFN-$\gamma$ (King et al, 1989; Zuckermann and Head, 1988). However, trophoblast can be killed by IL-2-stimulated natural killer cells (Drake and Head, 1989). NK cells also mediate spontaneous fetal resorption as shown by Baines et al and Kinsky et al in two different models. Baines showed that NK cells were the predominant infiltrating cells in the aborting feto-placental units in abortion-prone CBA X DBA/2 mice, and alloimmunization prevented this NK infiltration (Gendron and Baines, 1988). In Kinky's model, double stranded RNA (dsRNA), which is known to activate NK cells, was injected into normal mice and it caused high fetal resorption rates (Kinsky et al, 1990). Fetal resorption could also be induced by adoptive transfer of dsRNA-treated mouse spleen cells into pregnant mice. Treatment of these spleen cells with anti-asialo-GM1 antibody eliminated the adoptive transfer effect, suggesting that NK cells are directly involved in the induction of fetal resorption (Kinsky et al, 1990).

## 3. Tissue and Cellular Sources of Cytokines

Although most of the known regulatory cytokines are of lymphohematopoietic origin, a number of studies have localized production of them to non-lymphohematopoietic cells of the reproductive tract. Placental trophoblast cells, which share many characteristics with macrophage cells, have long been known as a source of cytokines. In the late 1970s, human placental cells were found to produce GM-CSF-like molecules because placental conditioned medium stimulated hematopoietic colony formation (Burgess et al, 1977). This was later confirmed by the report that GM-CSF mRNA is located in the spongiotrophoblast (Kanzaki et al, 1991). CSF-1, another cytokine from CSF family, is found in both the human and the mouse placenta (Pollard et al, 1987; Azouly et al, 1987). In addition, interleukin-6 (IL-6) (Tabibzadeh et al, 1989; Pollard et al, 1987), TNF-α (Yelavarthi et al, 1991), interleukin-1α (IL-1α) (Azoulay et al, 1987) and IL-2 (Boehm et al, 1989; Soubiran et al, 1987) have been reported to be produced by placental trophoblast cells.

Another cell type which is actively involved in cytokine production in the reproductive tract is the uterine epithelial cell. These cells were first reported as cytokine-secreting cells when they were shown to synthesize CSF-1 in the uterus of pregnant females (Daller et al, 1992). It is known now that a group of cytokines, including TNF-α (Yelavarthi et al, 1991), TNF-β (Tamada et al, 1990); GM-CSF (robertson and Seamark, 1992), IL-6 (robertson and Seamark, 1992), and leukemia inhibitory factor (LIF) (Bhatt et al, 1991), are produced by uterine epithelial cells.

Metrial gland cells also produce cytokines. The ones identified include: CSF-1, IL-1, and LIF (Croy et al, 1991).

In conclusion, there is a complex cytokine network at the maternal-fetal interface, contributed by the fetal tissues, the maternal immune system, and the reproductive tissues. This cytokine network plays an important role in mediating and regulating interaction between these tissues.

## D. Effects of Pregnancy on Certain Diseases.

It has been documented for a long time in medical practice that pregnancy changes the course of some infectious and autoimmune diseases, although this is mostly based on clinical observations and impressions, where proper controls are lacking.

### 1. Infectious Diseases.

Infectious diseases caused by intracellular pathogens such as intracellular bacteria, viruses, and parasites are associated with increased risk during pregnancy. Of the diseases induced by intracellular bacteria, tuberculosis has been most extensively observed with respect to the effect of pregnancy on the disease. In a book published in 1935, Jameson described some comments from "world-wide authorities" in the field (Jameson, 1935). Speaking from the experience of thousands of cases, most people considered that pregnancy is hazardous for the tubercular women: about 30-80% of pregnant women became worse. Many married tuberculous women traced the onset of their disease to a previous pregnancy (Jameson, 1935).

This conclusion has been confirmed by many reports (Logg et al, 1944, Rich et al, 1951, Nicholson et al, 1932, Weinstein et al, 1974). Of 200 tuberculosis specialists in Great Britain, U.S., France, Switzerland and elsewhere queried in a survey in the 30's, the "great majority" responded by stating that childbearing exerts a deleterious effect upon tuberculosis, and late pregnancy is the most dangerous period for the mother (Robinson et al, 1931). In addition, the size of the inflammatory lesions is exacerbated, and the risk of miliary tuberculosis is two to three fold higher in pregnant than in non-pregnant young female adults (Rich, 1951). As the result, in the early decades of this century when antibiotics were not available, the single most common indication for a therapeutically induced abortion was tuberculosis (Rich, 1951).

As with tuberculosis, pregnancy in lepers whose disease is caused by another intracellular bacterium, *Mycobacterium leprae*, has been observed to lead to a rapid development of the disease (Rich, 1951). Those who are cured or whose disease is under control with drug treatment either relapse or become resistant to the therapeutic agent during pregnancy (Louria et al, 1962).

Systemic fungal disease is another category of infection associated with an increased risk during pregnancy. Coccidioidomycosis has been reported to be worsened during pregnancy, causing high morbidity and mortality. In the general population of coccidioidomycosis patients, the incidence of dissemination is only 0.2%, but the dissemination rate is 20% among pregnant patients (Vaughan and Ramirez, 1951). In addition, coccidioidomycosis as a complication of pregnancy is as high as 91% during the third trimester (Vaughan and Ramirez, 1951). Furthermore, overall mortality of treated and untreated pregnant patients is 88%, compared with an estimated 50% mortality in untreated disseminated disease in the general population (Smith et al, 1946). This disease has been reported as a leading cause of maternal death in endemic areas (Smale et al, 1970).

Pregnancy also enhances the clinical severity of malaria. In the earlier decades of the century, it was recorded that parasitemia developed virulently, leading frequently to maternal death and to high rates of abortion, stillbirth, and premature delivery (Wickramasuriya et al, 1937). As a result, in the southern U.S. a half century ago, rural physicians routinely administered quinine to pregnant women (Rutmen et al, 1976).

In modern years, it has been frequently reported that morbidity and mortality of malaria is higher in pregnant women than in non-pregnant women (Bruce Chwatt, 1983; Gilles et al, 1969). One recent study analyzes more than 3000 cases from females residing in Keneba who were examined annually over a 15-year period. Malaria parasitemia among pregnant women is 31.8% compared to 25.9% in the non-pregnant population (McGregor, 1984). Even women living in holoendemic areas who are supposed to be protected by their immunity against severe malaria suffer the hazardous effects of repeated malaria attacks during pregnancy, particularly during their first pregnancy (Menon, 1972; Kortman et al, 1972). Comparable phenomena have also been observed in mice (van Zon et al, 1982).

Since the acquired immunodeficiency syndrome (AIDS) emerged in recent years, the effect of pregnancy on the progress of AIDS has drawn a lot of attention. Most reports have been based on clinical studies of a small number of patients without the proper controls. Thus, it remains uncertain whether or not pregnancy has adverse effect on the course of human immunodeficiency virus (HIV) infection.

There are studies which suggest that pregnancy accelerates the progression of AIDS. In the United States, the first five reported cases of pneumocystic pneumonia occuring during pregnancy were fatal. Some of the early AIDS cases in pregnant women are recognized because of the sudden onset and rapid progression of a severe, often fatal illness (Jensen et al, 1984; Minkoff et al, 1990).

The interval between the diagnosis of AIDS and death is shortened by pregnancy. Koonin and her colleagues reported that the mean interval for a group of 20 pregnant AIDS patients was 113 days (Koonin et al, 1989), while the mean length of survival of non-pregnant women patients from another report was 298 days (Rothenberg et al, 1987). Thus gestation appears to significantly accelerate the disease process of AIDS.

The accelerative effect of pregnancy on AIDS is also manifested by the loss of CD4 positive cells, which is one of the characteristic clinical features of AIDS (Melbye et al, 1986). In a prospective study of one hundred pregnant women at high risk for HIV infection, the level of CD4 positive cells was shown to fall during pregnancy and not recover during the postpartum period among HIV positive women. In contrast, the level of CD4 positive cells falls during pregnancy and rises rapidly just before delivery among HIV-negative women (Biggar et al, 1989).

Despite a number of reports that pregnancy may worsen the course of HIV infection, this hypothesis has been the subject of controversy. Gold et al (1982) pointed out that there have been many reports of groups of HIV-positive women who tolerate pregnancy with no adverse consequences. This was recently confirmed by Johnstone et al (1992). Their study shows that the clinical presentation, severity of the illness and laboratory findings are not obviously different in pregnancy, and the survival time of AIDS patients is not obviously reduced. They also suggest that the accelerating effect of pregnancy on HIV infection could be caused by a delay in the diagnosis (a low index of suspicion, symptoms of dyspnoea or fatigue falsely attributed to pregnancy), or by less aggressive investigation and treatment due to concern for the fetus. AIDS is a relatively new disease, and much needs to be learned about the status of the immune system and other factors that may determine whether disease progression occurs. Studies in progress comparing characterized groups of pregnant and nonpregnant infected women should help define the interaction between pregnancy and HIV.

In contrast to the infections reviewed previously, the infectious diseases caused by extracellular pathogens, in which CMI defense is not as important as humoral immunity, are less prominent in pregnancy (Weinberg, 1984).

## 2. Autoimmune Diseases.

Many autoimmune diseases have a peak incidence around puberty, so they often coexist with pregnancy. Class I MHC-associated diseases are generally male dominant, so they are less associated with pregnancy. On the other hand, class II MHC-associated diseases show a female preponderance. Among this category rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are best studied and documented for their association with pregnancy.

RA is a condition of unknown etiology that is manifested by inflammation of the synovial joints. Although the presence of IgM autoantibody is one of the characteristic manifestations, RA is not considered as an autoantibody-mediated autoimmune disease because transferring of patient's serum to normal recipient does not cause the disease (Varner, 1991; Klipple and Cecere, 1989). The ameliorating effect of pregnancy on the activity of RA has been repeatedly confirmed since Hench's observation in 1938 (Persellin, 1976; Hench et al, 1938; Kaplan and Diamond, 1965; Oka, 1953, Oka and Vainio, 1966). After an extensive review of the medical literature as well as with the addition of his own experiences, Persellin has reported that in a series of 308 pregnancies, RA substantially improves or resolves in 73% of patients and remains unchanged or worsened in 27% (Persellin, 1976). In a more recent literature review, Silva and Spector summarized 12 reports over a span of 45 years. Improvement or remission of RA during pregnancy are found in 77% (range 54%-95%) of 291 patients (Silva and spector, 1992).

The relief of symptoms is apparent from the first trimester in the majority of cases and tends to become more pronounced in the latter stages (Persellin, 1976). The remission is not long lasting: essentially every patient who experiences remission during pregnancy has a relapse in the postpartum period (Varner, 1991;

Silva and spector, 1992), and 50% have a recurrence by six weeks postpartum (Neely and Persellin, 1977).

In contrast to RA, SLE, a collagen vascular disease, is frequently exacerbated by pregnancy (Dubois et al, 1976). The clinical manifestations are variable. The presence of autoantibodies against a nuclear component is a hallmark of the disease (Tan, 1982). SLE can be transferred with patient's serum, so it is considered as an antibody-mediated autoimmune disease (Glass and Schur, 1977).

Given the remitting and relapsing nature of this disease, it has been extremely difficult to establish the interrelationship between pregnancy and SLE. There is no unanimous agreement about the impact of pregnancy on the frequency or extent of SLE exacerbation. When conception occurs during active SLE or in the presence of lupus nephritis, at least 50% of patients experience exacerbation (Varner, 1991; Hayslett and Lynn, 1980; Garsenstein et al, 1962). Pregnant patients with SLE who have no visceral involvement and who have not had exacerbation in the past year are most likely to have uneventful pregnancies. If exacerbations do occur in such pregnancies, they are usually mild (Hayslett and Lynn, 1980). Again the authors of most reviews suggest that pregnancy does not adversely affect the long-term course of SLE (Meehan and Dorsey, 1987; Gigor et al, 1979).

The association of other class II MHC-associated autoimmune diseases with pregnancy also are observed. Myasthenia gravis and autoimmune thrombocytopenic purpura are other autoantibody-mediated diseases, and their severity and activities are generally unaffected by coexistent pregnancy. On the other hand, disease activities are exacerbated in patients with scleroderma and polyarthritis (Plauche, 1983; Varner, 1991).

## E. The Maternal Immune Response Bias during Pregnancy

### 1. Depression of Cell-mediated Immunity (CMI) during Pregnancy

As reviewed in the previous section, pregnant women have decreased resistance to certain infectious diseases caused by intracellular pathogens, in which CMI plays an important defensive role. One mechanism explaining these clinical observations is that pregnancy is associated with depression of selective aspects of CMI. Because CMI is considered as the primary mechanism responsible for allogeneic graft rejection, depression of CMI can help to prevent rejection of the fetus (Weinberg, 1984).

This has been confirmed by studies in both humans and animals. In the early 40s, Lichtenstein demonstrated that the skin test reaction to tuberculin, which is considered a classical *in vivo* cell-mediated response, is diminished in pregnant females (Lichtenstein et al, 1942). This was later confirmed by Finn and his colleagues (Bhatt et al, 1991). It has also been found by different groups that paternal graft survival in pregnant mothers is modestly prolonged (Currie, 1970; Medawar et al, 1953; Anderson and Monroe, 1962).

But when four different types of antigens, including purified protein derivative (PPD), streptokinase-streptodornase (SKSD), *candida albicans* extract, and tetanus toxoid, were injected into pregnant women to test delayed type hypersensitivity (DTH), it was found that DTH response was normal among pregnant women (Hawes et al, 1981). This result was later contradicted (albeit in a different species) by the more sensitive experiments of Holland (Holland et al, 1984). He and his colleagues examined both the induction phase and effector phase of DTH response to burro red blood cells in mice. When primed lymphocytes and burro red blood cells were injected into mouse footpads, the swelling in pregnant mice was not different from non-pregnant control mice, indicating that the effector phase of DTH in pregnant mice is intact. Then burro red blood cells were injected into pregnant and non-pregnant mice for *in vivo* priming. Spleens were then harvested and the resulting cells were cultured with antigen. It was found that spleen cells from pregnant mice produced a significantly weaker DTH response. Thus the induction phase of the DTH response in pregnant mice is impaired. Similar results were obtained from rats (Amsden et al, 1987). The strain combination used was an unambiguously high responder, in which 100% of the females normally respond to the paternal antigens; there was no detectable CMI measured by the assays of DTH and IL-2 secretion in postpartum rats.

A number of *in vitro* experiments have documented the reduced cell-mediated responses. In the late 1970s, Hamilton and her colleagues, using lymphoid cell transfer experiments, demonstrated that the maternal cytotoxic response to allogeneic cells is depressed during pregnancy (Hamilton, 1976). When maternal CMI was further studied, it is found that the maternal spleen cell response to allogeneic tumors is depressed during pregnancy (Smith et al, 1978; Ferguson et al, 1982). In addition, the in *vitro* responses of maternal lymphocytes to phytohaemagglutinin (PHA), concanavalin A (Con A) and PPD are also suppressed (Alanen and Lassila, 1982).

### 2. Enhanced Antibody Production during Pregnancy.

As stated in previous sections, although alloreactive T cells are difficult to detect during pregnancy, alloantibodies are found regularly in pregnant females. Bell and Billington reported that the major alloantibody response induced by mouse pregnancy is the non-complement-fixing IgG1 subclass (Bell and Billington, 1980). Dillon and his colleagues reported that alloantibodies were detected in 48% of mice during or after a first pregnancy and in 82% of mice after a second pregnancy. An increased concentration of IgG1 was detected in multigravid sera, and the level of all other isotypes remained within normal limits (Sunderland et al, 1981). IgG1 isotype antibodies do not fix complement, and thus they are noncytotoxic antibodies. It is further confirmed by reports that the majority of pregnancy sera do not contain cytotoxic alloantibodies in mice (Dillon et al, 1988), in rats (Innes et al, 1988) and in humans (Cunningham et al, 1987). Voisin and Chaouat (1974) also showed that antibodies of IgG1 isotype bind to the placenta and do not fix complement. It has therefore been postulated that these antibodies may prevent possible complement-mediated cytotoxicity against placental cells and play a protective role in the placenta (Voisin, 1980).

In contrast to a depressed response to T cell mitogens, spleen cells from pregnant mice exhibit a normal response to lipopolysaccharide (Murgita, 1976). It has also been demonstrated that the humoral response is potentiated during murine pregnancy (Fabris, 1973; Sljivic et al, 1975). The number of immunoglobulin (Ig)-forming cells in mouse para-aortic lymph nodes (PALN) increases during pregnancy (Carter and Dresser, 1983; Mattsson and Mattsson, 1984) Recently the effects of pregnancy on the systemic humoral immune response were studied by Dresser (Dresser, 1991). He found that injection of sheep red blood cells into pregnant mice led to an enhanced systemic B cell response when compared with non-pregnant control mice, indicating that pregnancy has a potentiating effect on antibody production.

In conclusion, CMI is depressed while antibody production is enhanced during pregnancy. Thus the maternal immune response is biased towards antibody production and away from cellular immunity.

## F. Proposed Mechanisms for Biased Maternal Immune Response during Pregnancy

### 1. Pregnancy Serum

Before specific immunoregulatory factors were defined, sera from pregnant females were shown to be inhibitory of various immune responeses. In human studies, pregnancy sera diminish the release of lysosomal enzymes by cultured normal polymorphonuclear cells and decrease their phagocytosis of bacteria(Persellin and THoi, 1979). Pregnancy serum can also inhibit T cell proliferation in response to mitogens (Wajner et al, 1985) as well as mixed lymphocyte reaction (Gatti et al, 1973).

Similar results have been obtained from animal studies. Mouse pregnancy serum inhibits the lymphocyte transformation induced by mitogens and allogeneic spleen cells (Murgita, 1976). Allogeneic cytotoxicity is also suppressed by mouse pregancy serum (Smith, 1978). Large efforts have been made to identify the inhibitory factors circulating during pregnancy, which may be the products of the mother, the fetus or the placenta. Several factors have been studied and reported as being responsible for the inhibitory effect of pregnancy serum, but none of them is accepted without controversy. The principle ones described to date are as follows.

### 2. Progesterone

During the first ten weeks of human pregnancy, chorionic gonadotrophin stimulates the corpus luteum to secrete a high level of progesterone. Afterwards, the placenta becomes the major source of this hormone. Progesterone has well-known immunosuppressive properties. Administration of progesterone greatly prolongs the survival of skin allografts in animals (Moriyama and Sugawa, 1972; Siiteri et al, 1977). When further studied *in vitro*, progesterone suppresses neutrophil function and T cell mitogenic blastogenesis (Clemens et al, 1979), but it has been noticed that the concentration of progesterone needed to exhibit the above-cited effects is higher than those obtained from normal pregnant females (Perseilin et al, 1981). When Yagel and his group repeated the experiments, they found no suppression of maternal lymphocyte proliferation with up to 20 μg/ml progesterone, which is over a hundred-fold in excess of physiological concentration (Yagel et al, 1987).

It has been suggested then that physiological concentrations of progesterone could induce suppression factors (Yagel et al, 1987). Progesterone-induced blocking factor (PIBF) is one of them. This 34 kd protein is produced by lymphocytes from healthy pregnant women when incubated with physiological concentrations of progesterone (Szekeres Bartho et al, 1985). PIBF is capable of inhibiting the NK cell killing and the *in vitro* lymphocyte activation (Szekeres Bartho et al, 1985). PIBF is indeed found by ELISA in the circulation of normal pregnant women, and the serum level of PIBF decreases in women with preterm deliveries or miscarriages (Szekeres Bartho et al, 1989). Further characterization of this protein is necessory in order to understand its function during pregnancy.

### 3. $\alpha_2$-glycoprotein

Another candidate implicated as an immunosuppressive factor is pregnancy-associated $\alpha_2$-glycoprotein (PAG). PAG is a high molecular weight glycoprotein, the level of which increases approximately ten-fold during pregnancy and decreases to the insignificant level after delivery (Horne et al, 1982). PAG appears to be produced by estrogen-stimulated leukocytes (Horne et al, 1982). Increased concentrations also have been detected in patients with malignancies and some autoimmune diseases (Stimson, 1976; Peck et al, 1978; Horne et al, 1982). T lymphocyte activation by mitogens, antigens and allogeneic cells is suppressed by PAG in physiological concentrations (Stimson, 1976; Peck et al, 1978; Horne et al, 1982). However, according to Stimson and his colleagues, no correlation has been found between the concentration of PAG in individual pregnancy sera and the degree of inhibition of T cell activation by individual sera (Stimson, 1976). Furthermore, the inhibition of T cell activation only decreases from 60-70% to 40% when PAG is removed from pregnancy sera (Stimson, 1976). This suggests that PAG may have an inhibitory effect on T cell activation, but by itself is not sufficient to explain all the immunosuppressive effect of pregnancy serum.

### 4. α-Fetoprotein

α-Fetoprotein (AFP) is the fetal equivalent of serum albumin and is produced by the fetal liver and by the yolk sac. AFP increases in the maternal circulation during pregnancy. Murine AFP suppresses T cell activation (Hooper and Evans, 1989), but purified human AFP, even at concentration a hundred fold higher than noted in pregnancy sera, is not suppressive of T cell proliferation generated in the allogeneic mixed lymphocyte response (Charpentier et al, 1977). Moreover, removal of AFP from pregnancy serum does not abrogate the inhibitory effect of pregnancy serum on T cell activation (Stimson et al, 1980), so the relevance of AFP's inhibitory effect is very much in doubt.

### 5. Placental Extracts

The placenta is another source of an immunosuppressive factor(s) which has been extensively studied. Murine trophoblast cells from the ectoplacental cone and placental tissue inhibit the lymphocyte activation by mitogens and allogeneic cells (Kouttab et al, 1976; McIntyre et al, 1979). The results are confirmed by a report that murine trophoblast cells suppress allogeneic interaction in the mixed lymphocyte reaction and cell-mediated lympholysis (Persellin and Thoi, 1979). The same *in vitro* inhibitory effect has also been observed with supernatants of trophoblast cells and blastocytes (van Vlasselaer and Vandeputte, 1984), indicating the presence of secreted inhibitory factor(s). The production of the inhibitory factor(s) may be regulated by progesterone, because addition of anti-progesterone serum abolishes the immunosuppressive property of trophoblast supernatant (van Vlasselaer and Vandeputte, 1984)

When placental extracts are injected into mice, the recipient exhibits a delayed rejection of allogeneic tumor and a modification of antibody production (Duc et al, 1985). Although the overall hemagglutinating antibody production is not changed, the production of cytotoxic complement fixing antibodies is decreased, and the specific anaphylatic mast cell degranulating antibodies are increased (Duc et al, 1985), which is consistent with changes of Ig isotypes during pregnancy.

In conclusion, by combining *in vitro* and *in vivo* experiments, the placental substances can mediate the deviation of the immune response toward humoral response and away from cellular immunity.

### 6. Transforming Growth Factor-β

Transforming growth factor β (TGF-β) has drawn much attention to its immunosuppressive activity at the fetoplacental unit. TGF-β has a number of immunosuppressive effects. It inhibits natural effector cell activities, particularly the activation of NK cells, LAK generation, and macrophage activation by cytokines (Rook et al, 1986; Ding and Shevach, 1992; Kuppner et al, 1988) It also inhibits the growth of the trophoblast (Hunt and Orr, 1989).

A TGF-β-like molecule is found in decidua (Clark, 1990), para-aortic lymph nodes, that drain the uterus (Clark, 1990), and uterine epithelium (Cheng et al, personal communication). Very unstable TGF-β was also found in placental cells, which was explained as a binding to cells in the placenta of this TGF-β, which is produced elsewhere (Danielpour and Sporn, 1990). The production of TGF-β is reduced in abortion-prone DBA X CBA mice, and injection of monoclonal antibody against TGF-β2 leads to slightly increased abortion rates (Clark et al, 1991).

## G. Cytokine Secretion Patterns of T Helper Cells

### 1. TH1 and TH2 Cytokine Secretion Patterns

The functions of the immune system are carried out by different cell types. B cells, arising from bone marrow precusor cells, produce antibodies. T cells are derived from bone marrow and are educated in the thymus. T cells are further divided on the basis of function and surface markers. T cytotoxic cells kill target cells, and T helper (TH) cells mediate antibody production and cytotoxic killing. Only recently have functionally distinct types of T helper cells been described.

By studying mouse TH cell clones, two distinct TH cell types have been identified by the cytokines they secrete. TH1 cells produce IL-2, IFN-γ and lymphotoxin (LT); TH2 cells secrete IL-4, IL-5, IL-6, and IL-10, whereas both cell types produce GM-CSF and IL-3 (Fiorentino et al, 1989; Brown et al, 1989). These TH1 and TH2 cytokine patterns are stable and well established, and it has been strongly suggested that they occur and are important *in vivo* (Mosmann and Coffman, 1989). The same TH1 and TH2 cytokine patterns have also been established in humans (Paliard et al, 1988; Umetsu et al, 1988; Maggi et al, 1988). Furthermore, these cytokine profiles can be contributed by cells other than TH cells. CTL produces mostly TH1 cytokines (Mosmann and Moore, 1991), whereas cytokine profile of mast cells is considered to be TH2 (Moore et al, 1990; Paul, 1991; Brown et al, 1987; Razin et al, 1991). In addition, these two cytokine patterns are not the only two that exist. Other cytokine patterns of TH cells have also been identified, such as THp, THo and others (Street et al, 1990; Gajewski et al, 1988; Firestein et al, 1989; Kelso and Gough, 1988).

### 2. Functions of TH1 and TH2 Cytokines

TH1 cytokines basically mediate cellular immunity and TH2 cytokines help antibody production by B cells. When TH cells and antigen are injected into mouse footpads, only TH1 clones are able to produce antigen-specific DTH (Cher and Mosmann, 1987). This reaction involves IFN-$\gamma$ and results in Jone-Mote-like DTH, which is characterized by granulocyte infiltration with edema. TH1 cytokines have also been shown to enhance the generation of CTL *in vivo* (Erard et al, 1985; Simon et al, 1986).

Although IL-4 and IL-5 are reported to help to generate CTL (Pfeifer et al, 1987; Takatsu et al, 1987), TH2 cells do not mediate

DTH *in vivo*. Instead TH2 cytokines are excellent helpers for antibody production, among which, IL-4 and IL-5 are required for optimal proliferation of resting B cell and Ig production (Schumacher et al, 1988; Boom et al, 1988). IL-5 also is shown to stimulate the growth of large B cells. So IL-4 and IL-5 are the major helper factors for B cells, and high IL-4 and IL-5 levels are correlated with high antibody production.

TH-2 cytokines are also important in isotype regulation. IL-4 selectively induces Ig gene switching to the $\varepsilon$ and the $\gamma 1$ loci, which results in production of IgE and IgG1 (Killar et al, 1987; Stavnezer et al, 1988). In addition, IL-4 and IL-3 are mast cell growth factors (Mosmann et al, 1986), and IL-5 induces the proliferation and differentiation of eosinophils (Sanderson et al, 1986; Coffman et al, 1989). Thus TH2 cytokines are involved in the mediation of allergic reaction at different levels whereas TH1 cytokines help antibody production less efficiently, even suppressing B cell response under some conditions. But TH1 cytokines are involved in isotype switching. IFN$\gamma$ inhibits IL-4 induced Ig gene switching and induces switching to $\gamma 2\alpha$ locus, leading to the production of IgG2a (Snapper and Paul, 1987; Lebman and Coffman, 1988).

Overall, a strong TH1 response mediates several cytotoxic mechanisms, which effectively kill tumor cells and cells infected with intracellular bacteria, parasites, and virus. IFN$\gamma$, LT and complement-fixing IgG2a are also involved in cytotoxicity. In contrast a predominant TH2 response induces a high level of antibody production, and leads to an allergic response under extreme conditions.

### 3. Mutual Inhibition of TH1 and TH2 Cytokines

It has been known for a long time that antibody production and DTH response are mutually exclusive during a strong immune response (Parish, 1972). This phenomenon can now be explained by mutual inhibition of growth, differentiation, and cytokine production of TH1 and TH2 cells. IFN-γ inhibits proliferation of TH2 cells (Fernandez-Botran et al, 1988; Gajewski et al, 1988), and newly discovered IL-10 inhibits TH1 cytokine production without affecting proliferation.

Mouse IL-10 is an acid-sensitive 35-40 kd homodimer that is produced only by TH2 cells and not by TH1 cells (Fiorentino et al, 1989; Moore and Mosmann, 1991; Moore et al, 1992; Fiorentino et al, 1991b). In addition, IL-10 is also produced by $CD8^+$ T cells, $CD5^+$ B cells, B cell lymphomas (O'Garra et al, 1990), activated macrophages, (Fiorentino et al, 1991a) and keratinocytes (Enk and Katz, 1992). A striking characteristic of IL-10 is that the IL-10 gene shares a homologous sequence with an open reading frame in the Epstein-Barr virus BCRF1 (Moore et al, 1990). Interestingly enough, recombinant BCRF1 protein also inhibits IFN-γ synthesis by TH1 cells (Hsu et al, 1990), providing yet another example of viral capture of genetic control for purposes of escape from immune mechanisms.

### 4. *In vivo* Models for TH1/TH2 Cytokines

The mutual inhibitory TH cytokine regulation of antibody production and DTH response is not only demonstrated *in vitro* but is also confirmed *in vivo*, which has been best demonstrated in parasitic infection of animals and humans.

In mice, leishmania causes a spectrum of infectious disease ranging from self-healing cutaneous ulceration in resistant C57BL/6 mice to progressive visceral dissemination in susceptible BALB/c mice. When cytokine production by lymphocytes is examined, it is found that infected C57BL/6 mice mount a strong TH1 response, secreting IL-2 and IFN-γ, and infected BALB/c mice mount a strong TH2 response, producing IL-4 and IL-10 (Heinzel et al, 1991). Furthermore, the resistance against leishmania can be altered by injection of anti-IL4 and anti-IFN-γ into BALB/c and C57BL/6 mice respectively, which clearly indicates that the resistance to the disease is regulated by the ratios of TH1/TH2 cytokines (Heinzel et al, 1991; Belosevic et al, 1989). This type of mechanism is further confirmed by analogous experiments with toxoplasmosis in mice (Gazzinelli et al, 1991).

In human studies, ratios of TH1/TH2 cytokines are shown to correlate with resistance to leprosy (Yamamura et al, 1991). There are two clearly distinguished forms of the disease: tuberculoid and lepromatous. Tuberculoid leprosy is localized and is considered to be the resistant form. Lepromatous leprosy spreads and is considered to be the susceptible form. When *in situ* cytokine patterns were examined, it was found that messenger RNA (mRNA) for TH1 cytokines IL-2 and IFN-γ were predominant in tuberculoid lesions, while mRNA for TH2 cytokines IL-4, IL-5, and IL-10 were most evident in lepromatous lesions. Thus, susceptibility to the disease is correlated with the ratios of TH1/TH2 cytokines. In addition, the

TH1/TH2 balance recently has also provided a basis for understanding the progression of AIDS in humans (Shearer et al, 1992; Clerici and Shearer, 1993). The progression to AIDS is charaterized by loss of IL-2 and IFN-$\gamma$ production concomitant with transient increases in IL-4 and IL-10 (Shearer et al, 1992). Many seronegative HIV-exposed individuals generate strong TH1-type response to HIV (Clerici et al, 1992; Clerici et al, 1991). Thus, TH1-type responses may be immunoprotective, and a TH1 to TH2 switch caused by any signal would make the host more susceptible to HIV infection and progression to AIDS (Clerici and Shearer, 1993).

### H. Thesis Objectives and Rationale

This thesis was designed to investigate the mechanism of bidirectional cytokine interaction in the maternal-fetal relationship. As previously discussed, the maternal immune system recognizes the alloantigens expressed by the fetus and influences reproductive outcome. On the other hand, the maternal immune response is directed away from cell-mediated immunity towards humoral immunity because of the presence of the fetus. Although cytokines are known to play important roles in this interaction, currently known cytokines released at the maternal-fetal interface cannot explain the biased maternal immune response. Cell-mediated immunity and antibody production are at least partially regulated by TH1 and TH2 cytokines. Therefore, TH1 or TH2 cytokines may be produced at the interface and/or in the periphery, regulating local and/or systemic immune responses. This hypothesis was tested by the following approaches:

1. Examining the Systemic Production of TH1 and TH2 Cytokines.

Since maternal systemic immunity is altered, we examined whether TH1 and TH2 cytokines are produced systemically. Spleen and lymph node cells from pregnant mice were cultured with and without mitogen stimulation. Supernatants of these culture as well as sera from the same mice were tested for different cytokines. None of the TH1 or TH2 cytokines tested was found in these samples, with the exception of a small amount of IFN-$\gamma$ in stimulated spleen cells.

2. Examining the Local Production of TH1 and TH2 Cytokines at the Maternal-fetal Interface.

The feto-placental units from mice at different stages of gestation were cultured and lysed. The culture supernatants and cell lysates were tested for different cytokines. A group of TH2-biased cytokines were found in both culture supernatants and cell lysates.

3. Examining the Nature of the Cells that Secrete these Cytokines.

The cytokine-producing cells were localized by immunohistochemical staining with antibodies against the cytokines, and *in situ* hybridization with radiolabeled RNA probes. Another approach has been to study the cytokine production by feto-placental units from immunodeficient mice that do not have any funtional T, B or NK cells.

4. Examining the *in vivo* Functions of these Cytokines

A mouse model of spontaneous fetal resorption was studied to evaluate the importance of the TH2 cytokines *in vivo*. These mice have unusually high fetal resorption rates, which can be downregulated by various immunologically associated methods. The production of TH2-biased cytokines by fetoplacental units were compared among normal mice, fetal resorption-prone mice, and fetal resorption-prone mice manipulated to prevent fetal resorption.

# II. MATERIALS AND METHODS

## A. Mice.

BALB/c, C3H/HeJ and ICR mice (8-15 weeks) were obtained from the Laboratory Animal Breeding Unit at the University of Alberta (Ellerslie, Alberta, Canada). Mice were maintained in a conventional room, where investigators wore gloves, donned plastic boots, headcovers, masks and backfastening gowns in the room. The floors of the mouse rooms were washed with bleach weekly. There was no regular serological testing for common viral infections.

C.B-17 Scid/scid and scid/scid.bg/bg mice (8-15 weeks) were from the Isolation Unit of the Ontario Veterinary College, University of Guelph, Guelph, Ontario (Croy and Chapeau, 1990). These mice were maintained in a pathogen-free environment without prophylactic antibiotic treatment. All food, water and handling instruments were autoclaved, and all mouse manipulations were performed aseptically under laminar flow. Flora-defined sentinel CD1 mice were placed in the same environment and evaluated at regular intervals to monitor the colony for pathogen exposure. Sentinel mice were consistently seronegative in 17 tests for common rodent pathogens (Mouse Level II Complete Antibody Profile, Microbiological Associates, Rockvillle, MI). To screen for "leaky" SCID mice, an ouchterlony immuno-diffusion assay was used to measure murine Ig (Croy and Chapeau, 1990). Only "non-leaky" mice were used in the experiments. The bg/bg genotype was identified by staining blood films and detecting the presence of giant sudanophilic granules in 100% of their blood neutrophils (Croy and Chapeau, 1990).

Matings were conducted by caging the female mice overnight with the male mice and by checking the following morning for the presence of vaginal plugs. The day on which the plug was observed was considered to be day 0 of pregnancy.

## B. Preparation of Single Cell Suspensions.

### 1 Spleen and Lymph Nodes.

These organs were washed in ice-cold PBS, and minced between two glass slides. Tissue debris was removed by low speed centrifugation (40g for 20 seconds).

### 2 Placenta and Decidua.

At day 12 and day 18, embryo and placenta were removed from the uterus, and then dissected from one another. Day 12 placenta was further dissected into fetal placenta and decidual tissues with watchmakers forceps. The day 18 placenta was difficult to dissect, so the decidua was not separated from fetal placenta. The interface tissues were then rinsed with ice-cold HANKS medium, minced, and digested with 1mg/ml type I collagenase (Sigma, St. Louise, USA) and 25µg/ml DNase (Sigma). A total of 10 deciduas or placentas were placed in 10ml of HANKS medium, and the digestion was carried out on a vertically rotating mixer at 37°C for 45 minutes. Single cell suspensions were obtained by low speed centrifugation

(40g for 1 minute). The cells were washed 3 times with RPMI / 10% FCS before use.

## C. Tissue and Cell Cultures.

### 1 Tissue Culture.

The interface tissues were washed and kept in ice-cold HANKS medium. Then tissues were transferred to 24-well tissue culture plates (Falcon, Lincoln Park, USA), minced and cultured in RPMI 1640 / 10% FCS at a density of 1-10 embryos per ml of medium.

### 2 Cell Culture.

The cells were washed 3 times with RPMI / 10% FCS before they were put into 24-well tissue culture plates at 5 x $10^6$ /ml in RPMI / 10% FCS.

## D. Cell Lysate Preparation.

Cells were suspended in 1% Triton-X 100 in PBS at $5\times10^6$ cells/ml. Lysates were put on ice for 20 minutes, mixed well, and spun down (800g for 10 minutes) to remove cell debris.

## E. Cytokines and Their Antibodies.

Previously calibrated supernatants of Con A-activated HDK1, a TH1 clone, were used as standards for the IL-3 and IFN-$\gamma$ ELISAs. Calibrated supernatants from Con A-stimulated D10 cells, a TH2 clone, served as standards for the IL-4, IL-5 and IL-10 ELISA assays. These standards were used for ELISAs throughout this project. Therefore, levels of cytokines calculated using these standards are relative to each other and consistent. Monoclonal antibodies used in ELISAs are listed in table 2.1.

### F. ELISAs for Cytokines.

The double monoclonal antibody sandwich cytokine ELISAs were performed as described previously (Fiorentino et al, 1989). Flexible PVC microtitre plates (Dynatech Laboratories, Inc., Alexandria, USA) were coated with 100 μl of 1 μg/ml capture antibody in PBS for 30 minutes at 37°C, and blocked with 100 μl of 20% FCS and 0.1% Tween 20 in PBS for 30 minutes at 37°C. At least four serial two-fold dilutions of samples and mouse cytokine standards were made in RPMI / 10% FCS in triplicate and 50 μl were added to each well. In order to conserve the sample and to rule out nonspecific crosslinking, each dilution was transfered after 30 minutes incubation to a separate plate containing a different cytokine capture antibody. The process was repeated for the entire set of cytokine assays. The plates were washed three times with PBS containing 0.1% Tween 20 (PBST) and incubated with 50 μl biotinylated secondary antibodies (0.2μg/ml in PBST). After 60 minutes, the plates were washed four times with PBST and incubated with 75 μl of a 1:5000 dilution of streptavidin/horseradish peroxidase conjugate (The Jackson Laboratory, Bar Harbor, USA) in PBST plus 0.1% BSA for 45 minutes. Trays were washed five times

Table 2.1. Cytokine ELISAs

| ELISA | Detectability | Antibodies | | | |
|---|---|---|---|---|---|
| | | Clone | Species | Isotyppe | Reference |
| IL-3 | 125 pg/ml | 8F8 11 | Rat | IgG1 | Abrams et al, 1987 |
| | | 43D 11 | Rat | IgG2a | Abrams et al, 1987 |
| IL-4 | 38 U/ml | 1D 11 | Rat | IgG2b | Mosmann et al, 1990 |
| | | 24G 2 | Rat | IgG1 | Swain et al, 1990 |
| IL-5 | 125-313 pg/ml | TRFK4 | Rat | IgG2a | Schumacher et al, 1988 |
| | | TRFK5 | Rat | IgG1 | Schumacher et al, 1988 |
| IL-10 | 0.6 U/ml | SXC1 | Rat | IgM | Mosmann et al, 1990 |
| | | SXC2 | Rat | IgM | Mosmann et al, 1990 |
| IFN-γ | 0.6 U/ml | XMG1.2 | Rat | IgG1 | Cherwinski et al, 1987 |
| | | R4-6A2 | Rat | IgG1 | Spitalny et al, 1984 |

with PBST, and 100 µl of substrate (ABTS 1mg/ml, 0.003% $H_2O_2$, 44 mM $NaH_2PO_4 \cdot 7H_2O$, 28 mM citric acid) were added. After 60 minutes at room temperature, absorbance was measured at a wavelength of 405 nm on a VMAX microplate reader (Molecular Devices, Palo Alto, USA). The plates were also read at a reference of 490 nm in order to substrate backgroud. The values falling in the linear range of the standard curve derived by titrating cytokine standards were used to calculate the concentration of cytokines in units or picograms per ml. The detectability of each ELISA is listed in table 2.1.

It is possible that a positive signal in the ELISA could be due to nonspecific cross-linking of the capture and secondary antibodies. One way to exclude this possibility is to use the same antibody as both capture and secondary antibodies. This was routinely done, with negative results. This is particularly important for serum samples because of the presence of mouse anti-rat Ig natural antibodies in the serum.

### G. Reverse Phase Fast Protein Liquid Chromatography.

The ProRPC HR 5/2 column was connected to a LCC Liquid Chromatography controller (Pharmacia Fine Chemicals AB, Sweden). Water was used as an inorganic eluent, and acetonitrile as an organic eluent. Both eluents were acidified with 0.1% trifluoroacetic acid. Supernatants in a 1 ml volume were acidified and eluted at a flow rate of 0.7 ml/min with the concentration of acetonitrile increasing at 1% per minute. One minute fractions were collected into microtubes frozen at minus 70°C overnight, lyophilized, and reconstituted with 0.7 ml RPMI.

### H. IFN-γ Synthesis Inhibition Bioassay of IL-10.

IL-10 inhibits the IFN-γ production by IL-2 stimulated spleen cells. This forms the basis of this assay. 50 µl aliquots of samples to be tested were plated in 96-well flat-bottom microplates (from Evergreen Scientific, CA, USA ). A total of $10^6$ virgin mouse spleen cells in 50 µl of RPMI / 10% FCS, with 50 µl of 20 µg/ml monoclonal anti-IL-10 antibody (SXC1) or normal rat IgG control, were added to the wells. The plates were incubated in 5% $CO_2$ at 37°C for 2 hours before 50 µl of recombinant mouse IL-2 (0.4% of E. coli IL-2 transfectant supernatant) was added into each well. The final volume of each culture was 200 µl, and the plates were incubated at 37°C for 24 hours. The supernatants were collected and tested for the amount of IFN-γ using the above-mentioned ELISA. Recombinant mouse IL-10 was used at 40 U/ml as a positive control, and the medium only as a negative control.

### I. Con A Stimulation.

Cells were cultured at 5 x $10^6$ cells/ml in RPMI / 10% FCS with 5 µg/ml Con A. The supernatants were collected after 24 hours of culture.

### J. Frozen Tissue Preparation for *in situ* Hybridization.

The mice were perfused transcardically with 50 ml cold saline and 50 ml 4% paraformaldehyde in phosphate buffer (PBS). The uteri were dissected out and immersion fixed in the 4% paraformaldehyde for 4 hours at 4°C. The tissues were cryoprotected by immersion for 2 hours in each of 12%, 16% and 18% sucrose in PBS. Then the tissues were frozen in liquid nitrogen with embedding medium (OCT compound, Miles Inc. Elkhart, USA), and stored at -70°C. 10 µm tissue sections were cut on the cryostat and collected on slides which had been coated with chrom alum-gelatin adhesive. (Slides were rinsed with 1% HCl/70% ethanol and 95% ethanol. Then air dried slides were dipped in chrom alum-gelatin gel contained 0.4% gelatin and 0.04% chromium potassium sulfate. Slides were dried overnight and used two weeks later). After air drying at room temperature (RT) for 4 hours, slides were fixed with 4% paraformaldehyde in phosphate buffer for 20 minutes at RT. Then the slides were rinsed in PBS and treated with 20 µg/ml of proteinase (Boehringer Mannheim, Germany) in TE buffer for 7.5 minutes at RT. The slides were then rinsed with PBS, postfixed with 4% paraformaldehyde, dehydrated for 5 minutes in graded ethanols of 70%, 90%, 95% and 100%. Slides were air dried and proceeded to *in situ* hybridization.

### K. Radiolabeling of RNA Probes

Bluescript SK$^-$ (2.95 kilobase pair) which contains a 582 base pair (bp) fragment of mouse IL-10 cDNA (pIL-10δ) was kindly provided by Dr. J. F. Elliott of the University of Alberta. The PCRamplified pIL-10δ fragment was originally cloned into bluescript SK- at a NotI/Xba site between the T3 and T7 RNA promoters. Transfection of BW5147 cells with plasmid pJFE14 containing the pIL-10δ fragment was known to lead to the successful expression of the protein of IL-10.

The plasmid was linearized by the digestion with Xba I (anti-sense) and Not I (sense). Riboprobes transcription was performed with the *in vitro* transcription kit (Ambion Inc., Austin, Texas, USA). 1 μg linearized plasmid was incubated with 0.5 mCi/ml $^{35}$S-CTP (1200 Ci/mmol, NEN, Du Pont Canada Inc., Ontario, Canada), 0.5 mM each of three other nucleotides (ATP, GTP, UTP), RNasin, 10 mM DTT, 0.2 U/ml RNA polymerases T3 (anti-sense) or T7 (sense) for 1 hours at 37°C. Approximately $10^7$ cpm were incorporated into RNA probes per μg of template DNA.

## L. *In situ* hybridization.

The prehybridization mixture was composed of 50% formamide, 5x PIPES, 5x Denhardt's solution, 0.2% sodium dodecyl sulfate, 100mM dithiothreitol, 125 μg/ml salmon sperm DNA, 125 μg/ml herring sperm DNA, and 250 μg/ml yeast tRNA. 1 ml prehybridization mixture was added to each slide and slides were incubated at 37°C for 1 hour in a humidified chamber. Then labeled RNA probes were added to the prehybridization mixture. 100 μl of the hybridization mixture was loaded on each slide and hybridization was performed at 45°C for 15 hours in the humidifier chamber. After hybridization, the slides were washed with 14 mM βmercaptoethanol / 4x SSC (0.6 M sodium chloride and 0.06 M sodium citrate, pH7.0), and treated with 10 μg/ml RNase (Boehringer-Mannhein, Germany) in 0.5 M NaCl, 10 mM Tris-Cl (pH7.6), 1mM ethylene-deamine tetraacetic acid at 37°C for 30 minutes. The slides were then washed with the same buffer without RNase at 37°C for 30 minutes, with 2x SSC once at RT, with 0.1x SSC at 45°C and air dried.

Autoradiography was performed at -20°C for 10-14 days after coating the slides with autoradiographic emulsion (Kodak NTB-2, Kodak, Rochester, USA). The slides were developed with Kodak developer D19 for 2 minutes, rinsed with water for 30 seconds, and fixed with Kodak fixer for 5 minutes. Then slides were washed with water for 30 minutes before being counterstained with hematoxylin.

### M. Ovine trophoblast protein (oTP) treatment.

Recombinant oTP was obtained from TRANSGENE (Transgene SA, Cedex, France). 0.6 μg of recombinant oTP was injected i.p. on day 5 of pregnancy.

### N. Repetition of Experiments.

In general, all experiments were repeated 3-5 times to test for consistency, and this is indicated in the Figure and Table legends. If no indication is there, the experiment was done once.

# III. RESULTS: DETERMINATION OF CONSTITUTIVE CYTOKINE PRODUCTION AT THE MATERNAL-FETAL INTERFACE AND IN THE PERIPHERAL LYMPHOID ORGANS

The fundamental question underlying this investigation is: what is the dominant immunoregulatory cytokine secretion profile at the maternal-fetal interface and in the peripheral immune system during pregnancy? We started by examining both reproductive and systemic immune tissues for spontaneous cytokine production at different stages of mouse gestation. In particular, we examined subtype-specific cytokines, which included the TH2-specific cytokines IL-4, IL-5 and IL10, and the TH1 specific cytokine IFN-$\gamma$. In addition, we examined IL-3 because of its known role in preventing spontaneous fetal resorption (Chaouat et al, 1990).

There are basically two types of assays for cytokines: bioassay testing of the biological functions of cytokines and ELISA that detects the presence of cytokines by antibodies.

Bioassays measure the proliferation or metabolism of the cytokine-specific indicator cells in response to cytokines. These bioassays are sensitive under the condition that there is only one cytokine in the sample tested. Tissues at the maternal-fetal interface have been known to produce different cytokines as well as substances which inhibit the proliferation of many indicator cells and make the bioassays insensitive.

Due to progress in the production of monoclonal antibodies against cytokines, double monoclonal antibody sandwich ELISAs are increasingly popular. They are sensitive and specific, rendering them especially useful for samples containing multiple cytokines. In addition, ELISA can be used with samples which contain toxic substances. For instance, cell lysates, which kill the indicator cells and could not be tested in bioassays, can be tested by ELISA, although some proteases can even inhibit ELISA. Therefore most of our work on the characterization of cytokines were done using ELISA. The positive results were further confirmed by other assays.

Previous work in Dr. Wegmann's and other laboratories have shown that cytokines of the CSF family are present in the supernatants of interface tissues. Although most studies used the 24 hour culture as the standard method to collect supernatant, the kinetics of cytokine secretion into the supernatant by tissues or cells in culture within 24 hours is as yet undefined. We therefore studied the kinetics of cytokine production by decidual and placental cells from the fetoplacental units, as well as by uterine draining para-aortic lymph node (PALN) cells, peripheral mesenteric lymph node (MLN) cells and spleen cells.

## A. Kinetic Study of Cytokine Secretion by Cultured Feto-placental Units.

Initially, tissues from different pregnant mice were pooled to obtain enough sample for testing. Pregnant mice were sacrificed on days 6, 12 and 18, representing early, middle, and late gestation. Due to the nature of the developing feto-placental unit, cultures were set up differently at each of these stages. On day 6 the embryo cannot be easily separated from the placenta, so the combined embryo and placenta was minced and small pieces of tissue were cultured at a concentration of 10 conceptuses / ml in RPMI containing 10% FCS. On day 12, decidual and fetal placental tissues were separated by dissection, digested with 1 mg/ml collagenase and 25 μg/ml DNase at 37°C for 45 minutes, and cultured separately in RPMI+10% FCS. On day 18, the decidual layer cannot be separated from the fetal placenta, so the whole placenta was digested and single cells cultured with RPMI+10%FCS.

To examine the parameters of cytokine release at day 6, day 12 and day 18, supernatants were collected at 2, 4, 6, 8, 10, 12 and 24 hours and tested for IL-3, IL-4, IL-5, IL-10 and IFN-γ using double monoclonal antibody sandwich ELISAs. These experiments were repeated three times and comparable results were obtained each time. The representative results are shown in Figures 3.1-3.5. Figures 3.1-3.4 show that IL-3, IL-4, IL-5 and IL-10 were present in the supernatants of fetal-placental units from days 6, 12 and 18 of pregnancy. In contrast, IFN-γ was detected only from tissues and cells at days 6 and 12 (Figure 3.5). For day 6, the peak concentrations of IL-3, IL-4, IL-5, IL-10 and IFN-γ were found at 6-12 hours. For days 12 and 18 supernatants, the levels of IL-3, IL-4, IL-5 and IL-10 were maximal at 2 hours, the earliest time point tested in this experiment. These different cytokine release patterns on different days of gestation were consistently seen throughout these experiments. The reader must keep in mind that the day 6 tissues were of necessity prepared in a different way from days 12 and 18, and this could account for some of the differences in the kinetic curves.

Figure 3.1. Kinetics of IL-3 secretion in cultures of tissues and cells from day 6, day 12 and day 18 pregnant mice.

Day 6 conceptuses from ICR x ICR mice (Figure 3.1a) were minced and cultured at 10 conceptuses / ml in RPMI+10% FCS. Day 12 decidual and placental tissues (Figure 3.1b), and day 18 placental/decidual tissues (Figure 3.1c) were digested with 1mg/ml collagenase and 25 µg/ml DNase, the single cells were then washed three times in RPMI+10% FCS and cultured at $5x10^6$ cells/ml in RPMI+10% FCS. Freshly isolated spleen cells, MLN cells, and PALN cells were washed three times with PBS and cultured at the same cell density in RPMI+10% FCS. Supernatants were collected at different time points and tested for IL-3 by double monoclonal antibody sandwich ELISA. Eleven day 6 pregnant mice, eight day 12 mice, and eight day 18 mice were used for these experiments. The tissues from the conceptuses and the mothers were pooled from all pregnant mice of the same day. The results represent the mean and standard deviation of triplicate assays of the same samples. Comparable results were obtained in 2 other experiments (data not shown).

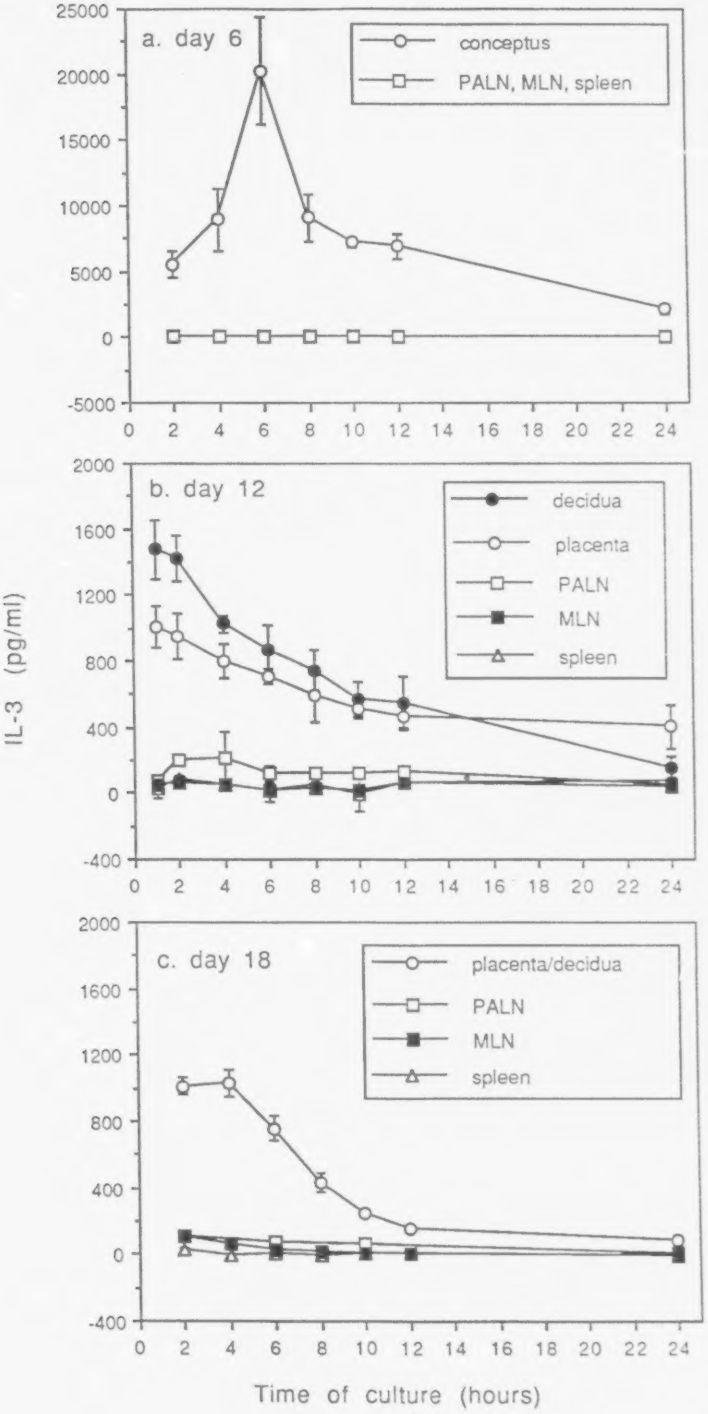

a. day 6
conceptus
PALN, MLN, spleen
25000
20000
15000
10000
5000
0
-5000
b. day 12
decidua
placenta
PALN
MLN
spleen
2000
1600
1200
800
400
0
-400
c. day 18
placenta/decidua
PALN
MLN
spleen
0 2 4 6 8 10 12 14 16 18 20 22 24
IL-3 (pg/ml)
Time of culture (hours)

Figure 3.2. Kinetics of IL-4 secretion in cultures of tissues and cells from day 6, day 12 and day 18 pregnant mice.

The pooled day 6 (Figure 3.2a), day 12 (Figure 3.2b) and day 18 (Figure 3.2c) samples were collected as described in the legend for Figure 3.1 and tested for IL-4 using ELISA. The results represent the mean and standard deviation of triplicate assays of the same samples. Comparable results were obtained in 2 other experiments (data not shown).

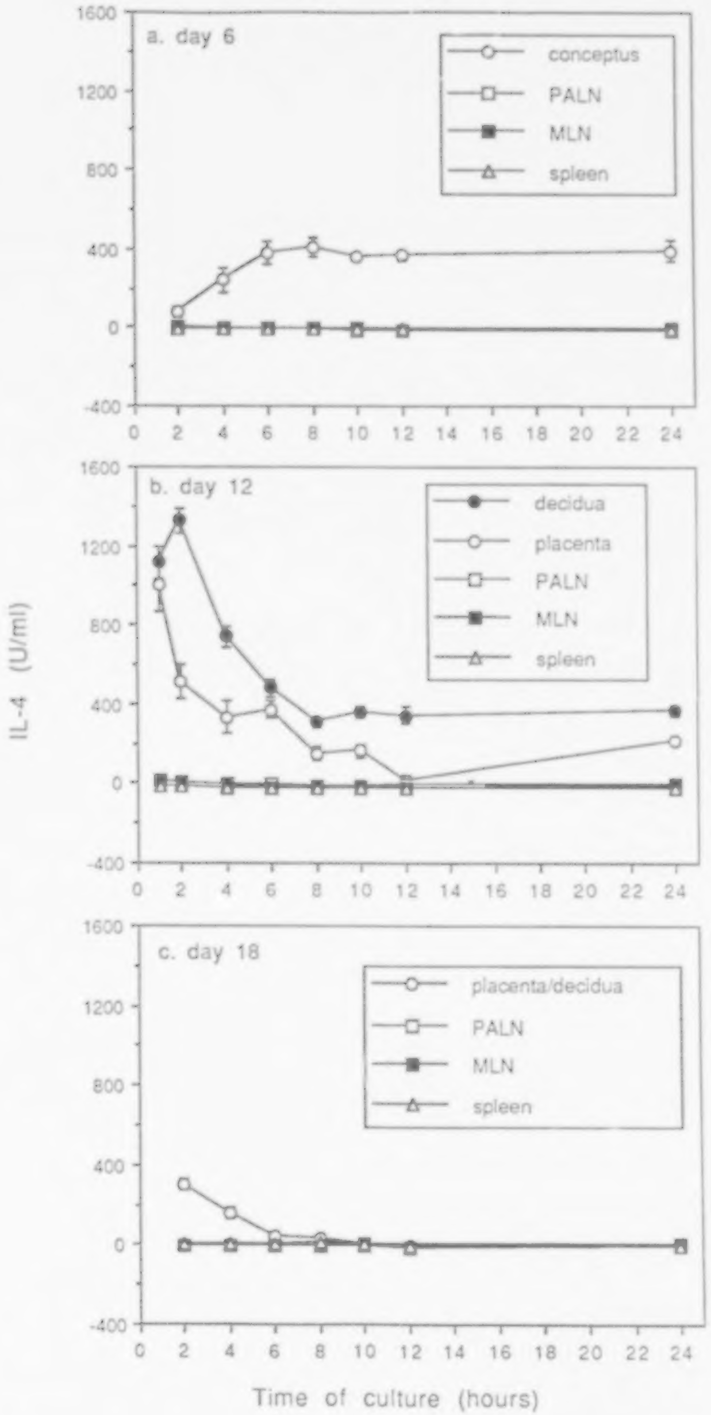

a. day 6
conceptus
PALN
MLN
spleen
b. day 12
decidua
placenta
PALN
MLN
spleen
c. day 18
placenta/decidua
PALN
MLN
spleen
IL-4 (U/ml)
1600
1200
800
400
0
-400
0
2
4
6
8
10
12
14
16
18
20
22
24
Time of culture (hours)

Figure 3.3. Kinetics of IL-5 secretion in cultures of tissues and cells from day 6, day 12 and day 18 pregnant mice.

The pooled day 6 (Figure 3.3a), day 12 (Figure 3.3b) and day 18 (Figure 3.3c) samples were collected as described in the legend for Figure 3.1 and tested for IL-5 using ELISA. The results represent the mean and standard deviation of triplicate assays of the same samples. Comparable results were obtained in 2 other experiments (data not shown).

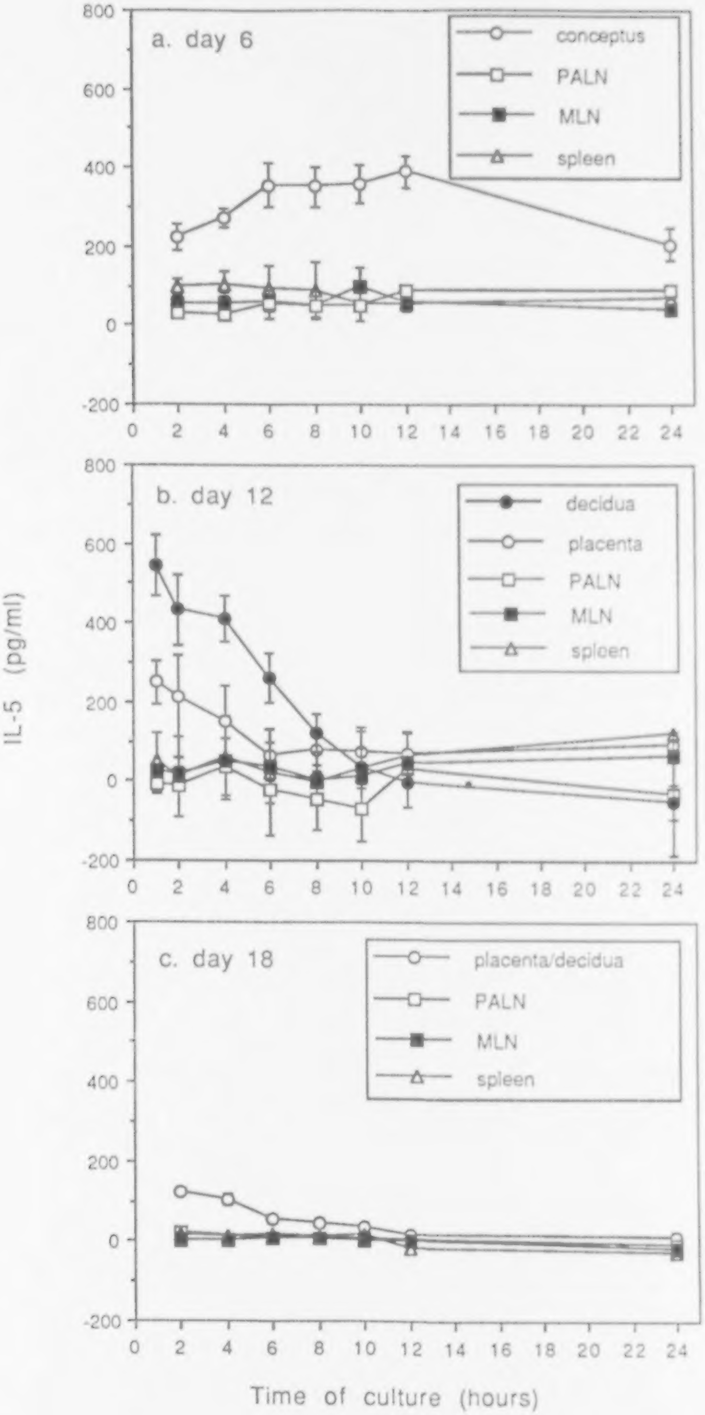

a. day 6
conceptus
PALN
MLN
spleen
b. day 12
decidua
placenta
PALN
MLN
spleen
c. day 18
placenta/decidua
PALN
MLN
spleen
800
600
400
200
0
-200
0 2 4 6 8 10 12 14 16 18 20 22 24
IL-5 (pg/ml)
Time of culture (hours)

Figure 3.4. Kinetics of IL-10 secretion in cultures of tissues and cells from day 6, day 12 and day 18 pregnant mice.

The pooled day 6 (Figure 3.4a), day 12 (Figure 3.4b) and day 18 (Figure 3.4c) samples were collected as described in the legend for Figure 3.1, and tested for IL-10 using ELISA. The results represent the mean and standard deviation of triplicate assays of the same samples. Comparable results were obtained in 2 other experiments (data not shown).

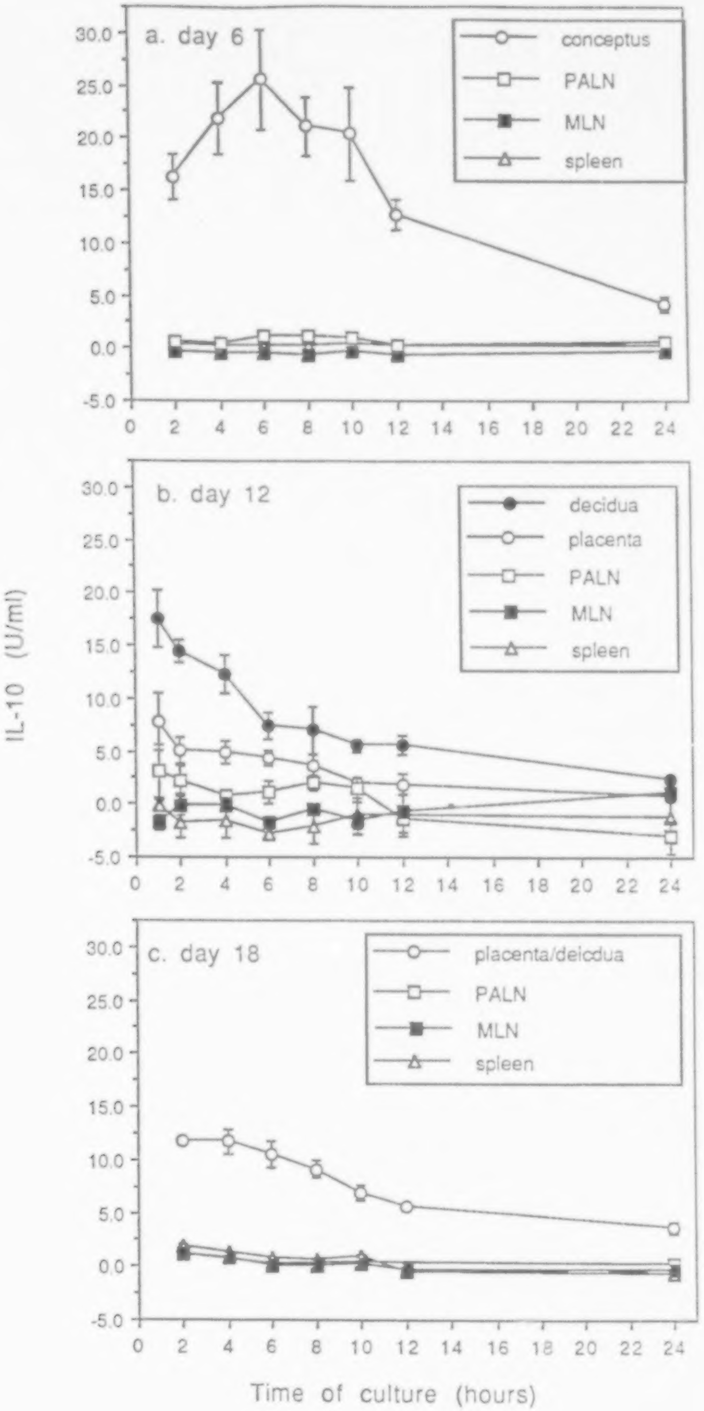
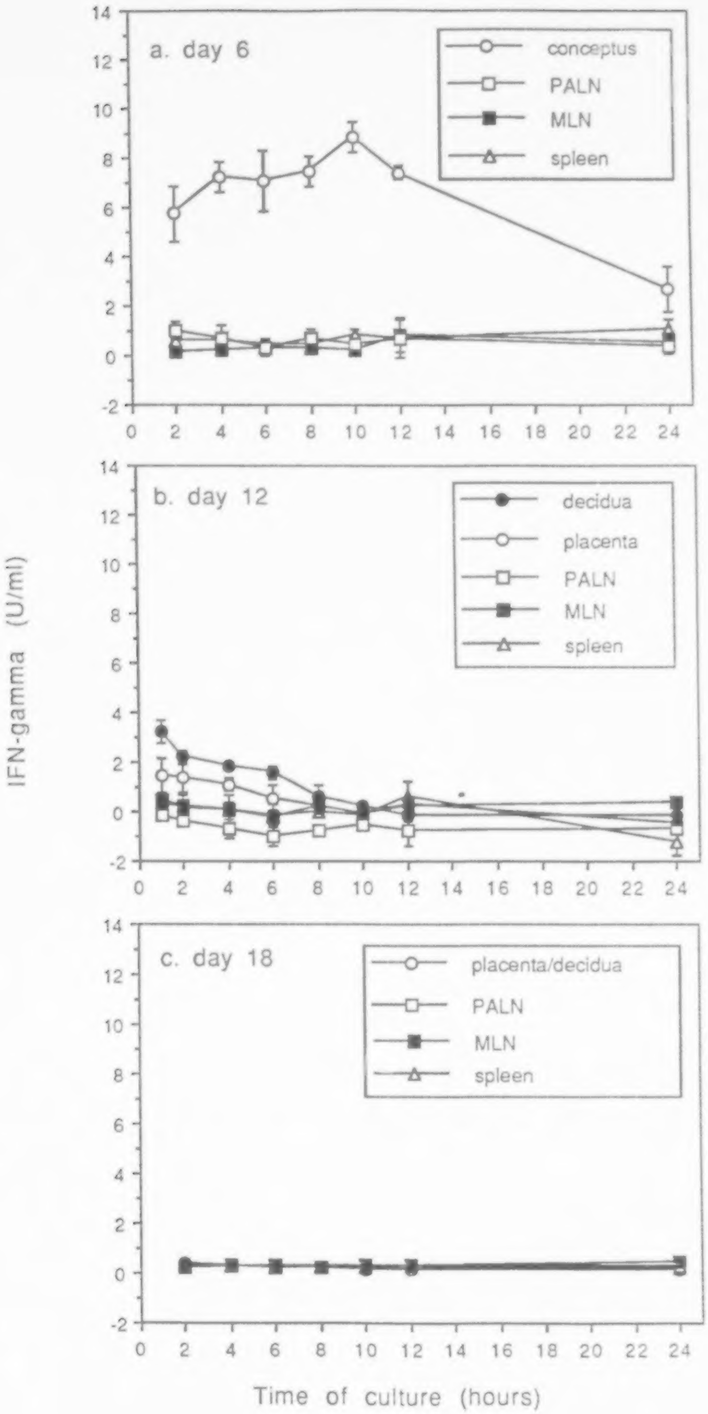

a. day 6
conceptus
PALN
MLN
spleen
b. day 12
decidua
placenta
PALN
MLN
spleen
c. day 18
placenta/deicdua
PALN
MLN
spleen
IL-10 (U/ml)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time of culture (hours)

Figure 3.5. Kinetics of IFN-γ secretion in cultures of tissues and cells from day 6, day 12 and day 18 pregnant mice.

The pooled day 6 (Figure 3.5a), day 12 (Figure 3.5b) and day 18 (Figure 3.5c) samples were collected in the same manner as described in the legend for Figure 3.1, and tested for IFN-γ using ELISA. The results represent the mean and standard deviation of triplicate assays of the same samples. Comparable results were obtained in 2 other experiments (data not shown).


a. day 6
conceptus
PALN
MLN
spleen
b. day 12
decidua
placenta
PALN
MLN
spleen
c. day 18
placenta/decidua
PALN
MLN
spleen
IFN-gamma (U/ml)
Time of culture (hours)
-2
0
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14
16
18
20
22
24

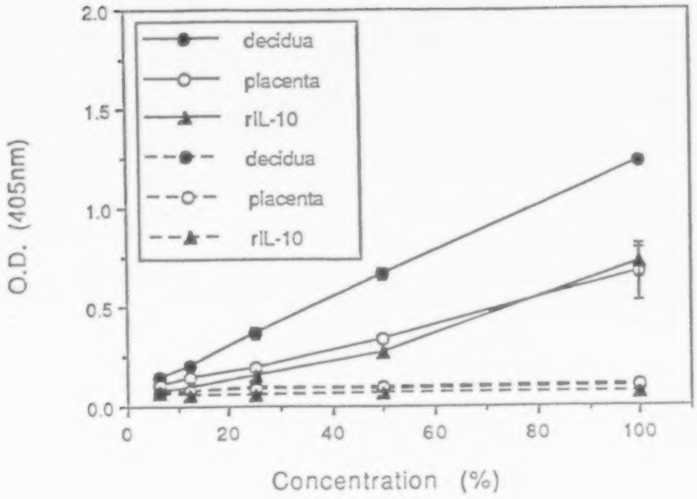


Figure 3.6. Control experiment for IL-10 ELISA.

Day 12 decidual cell supernatant, placental cell supernatant and recombinant IL-10 were tested in two different ELISAs. The solid lines (–) represent the results of conventional ELISA, in which the capturing antibody and the detecting antibody were two different antibodies. The dotted lines (--) represent the results of control ELISA, in which the capturing antibody and the detecting antibody were the same antibody. The results represent the mean and standard deviation of triplicate assays of same samples. This experiment was repeated three times with comparable results (data not shown).

In order to exclude the possibility that crosslinking of capture and secondary antibodies caused false positive signals, e.g. by mouse anti-rat Ig natural antibodies, the same capture and secondary antibodies were used in control ELISAs for most of positive samples. Figure 3.6 shows the results of conventional and control IL-10 ELISAs, which was a typical example of these control experiments. With different capture and detecting antibodies, day 12 decidual and placental cell supernatants, as well as recombinant IL-10 exhibited good titration curves, while there were no positive signals in the same samples when the same antibody was used as both capture and detecting antibodies.

None of the supernatants of cultured spleen, PALN, or MLN cells contained significant levels of IL-3, IL-4, IL-5, IL-10 or IFN-γ in any of the experiments performed.

In summary, we established the kinetics of different cytokine production from different tissues using ELISA. We consistently detected a group of TH2-type cytokines, including IL-4, IL-5 and IL-10, along with IL-3 in the supernatants of cells at the maternal-fetal interface in all three trimesters of pregnancy. IFN-γ was also found in the supernatants of these cells, maximally at day 6 with declining levels thereafter. None of these cytokines was detected in the supernatants of systemic lymphoid cells.

### B. Confirmation of the Presence of IL-3 and IL-10 by Independent Assays.

Although the control ELISAs were routinely performed to exclude false positive results, we still sought to independently verify the presence of different cytokines in the samples tested.

The fast protein liquid chromatography reverse phase column (FPLC-RPC) is a commonly used method to separate and isolate cytokines of different hydrophobicity, and the FPLC-RPC profiles of most of the cytokines we studied were known. Taking advantage of this, we compared the FPLC-RPC profiles of the cytokines found at the interface cell supernatants with those of standard T cell-secreted cytokines. 1 ml day 12 decidual cell supernatant was acidified with 0.1% trifluoroacetic acid, loaded on the column, and eluted by a increasing gradient of acetonitrile. Each fraction was lyophilized, reconstituted with the same volume of RPMI medium, and tested by different cytokine ELISAs. The experiments were repeated five times and similar FPLC-RPC profiles were obtained. The results of representative experiments are shown in Figures 3.7 and 3.8.

Figure 3.7 shows the IL-3 FPLC-RPC profile of day 12 decidual cells side by side with that from D10 cells, which is a cloned TH2 cell line. D10-derived IL-3 was eluted around 32-37% of acetonitrile, as expected from previous reports (Ihle, 1985). IL-3 from decidual supernatant was eluted at the same concentration range of acetonitrile. This indicates that the positive signal detected in the decidual cell supernatant by ELISA is most likely IL-3 because it displays the expected hydrophobicity.

Control T cell-derived IL-4, IL-5 and IFN-γ all showed the expected HPLC-RPC profile (Ohara et al, 1985; Takatsu et al, 1985;

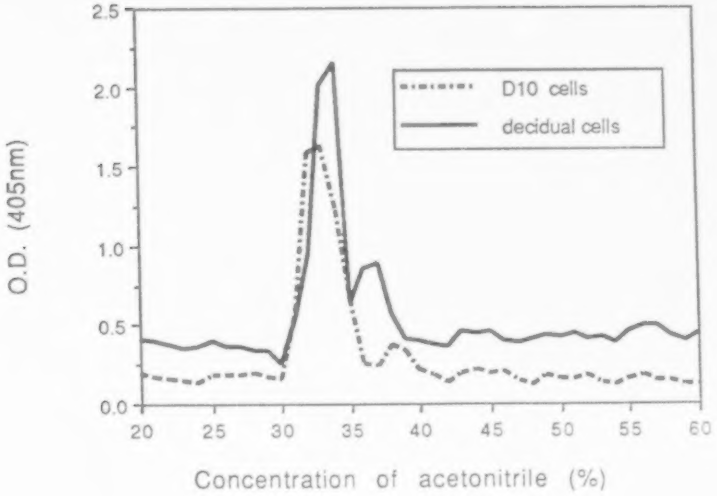
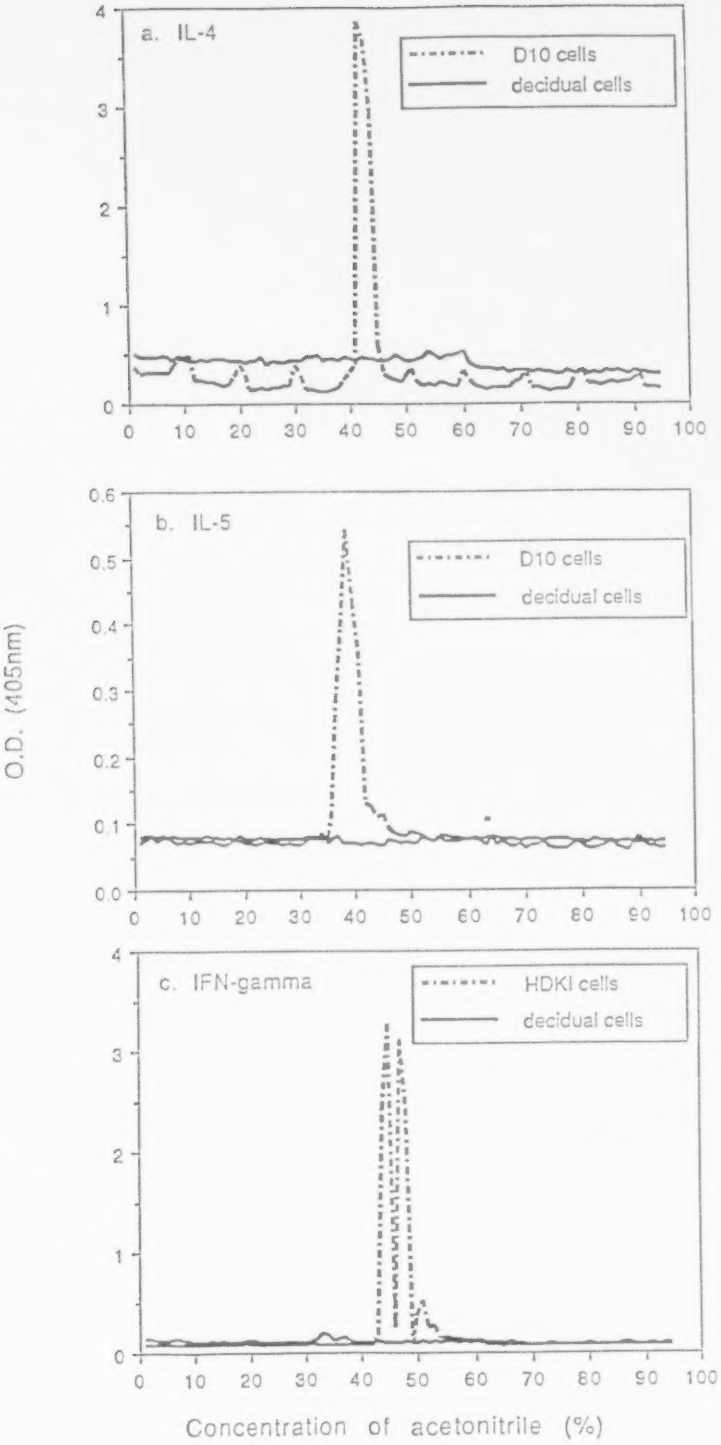


Figure 3.7. Decidual and control (D10) IL-3 fractionated by reverse phase fast protein liquid chromatography.

One milliliter of decidual and D10 supernatant was acidified with 0.1% trifluoroacetic acid, injected into the column, and eluted with increasing concentrations of acetonitrile. The fractions were collected, lyophilized, reconstituted with the same volume of RPMI, and tested using the IL-3 ELISA. The decidual cell supernatant contained 10% FCS and the D10 cell supernatant was serum-free. Comparable results were obtained in 4 other experiments (data not shown).

Figure 3.8. Decidual and T cell-derived IL-4, IL-5 and IFN-γ fractioned by reverse phase fast protein liquid chromatography.

Supernatants of decidual cells and T cells were fractiond in the same manner as described in the legend for Figure 3.7. The supernatant of D10 cells was used as an known source of T cell-derived IL-4 (Figure 3.8a) and IL-5 (Figure 3.8b). The supernatant of HDK1 cells was used as T cell-derived IFN-γ (Figure 3.8c). The fractions were tested for indicated cytokines using ELISAs. The decidual cell supernatant contained 10% FCS. D10 and HDK1 cell supernatants were serum-free. Comparable results were obtained in 4 other experiments (data not shown).


a. IL-4
D10 cells
decidual cells
b. IL-5
D10 cells
decidual cells
c. IFN-gamma
HDKI cells
decidual cells
O.D. (405nm)
Concentration of acetonitrile (%)
0
1
2
3
4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100

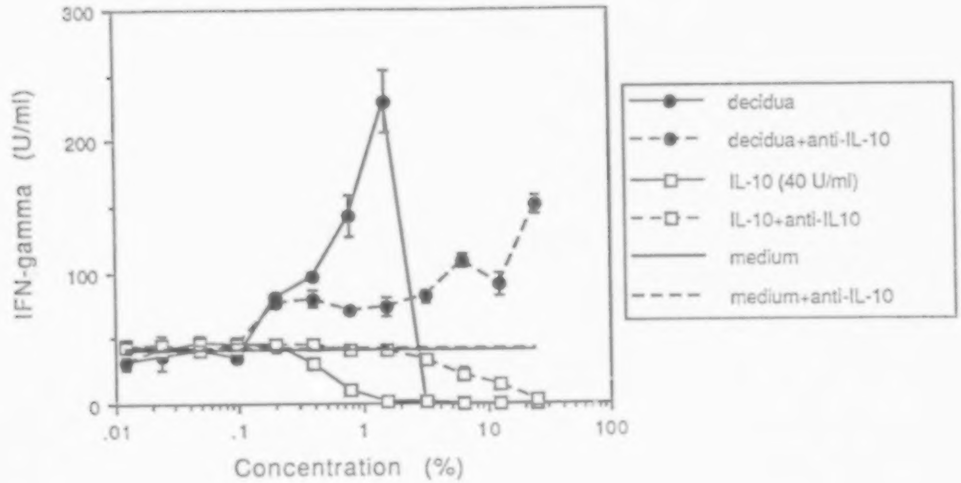


Figure 3.9. Decidual cell supernatant·IL-10 as detected by an IFN-$\gamma$ synthesis inhibition bioassay.

Titrated day 12 decidual cell supernatant and recombinant IL-10, with and without monoclonal antibody against IL-10 (sxc-1), were cultured with spleen cells from normal BALB/c mice ($10^6$ cells/ml) in the presence of IL-2 (0.1% of E. coli IL-2 transfectant supernatant). RPMI / 10% FCS medium was used as negative control. Supernatants were collected after 24 hour of culturing and tested for the amount of IFN-$\gamma$ by an ELISA. Results represent the mean and standard deviation of triplicate assays of same samples. Comparable results were obtained in 3 other experiments (data not shown).

Nagata et al, 1991) (figure 3.8). However, when we subjected decidual supernatants to this analysis, a positive signal was not obtained in the subsequent ELISA, indicating that the levels of these cytokines are too low to confirm in this manner (figure 3.8).

The acid treatment required for the reverse phase column separation destroys IL-10 activity (Moore and Mosmann, 1991), so IL-10 cannot be recovered after HPLC-RPC separation. Therefore, the presence of IL-10 in the decidual cell supernatants was confirmed by an IL-10 bioassay with a blocking antibody.

IL-10 inhibits IFN-γ production by many cell types, including IL-2 stimulated normal spleen cells. This function of IL-10 forms the basis of an IL-10 bioassay. Four experiments were done to evaluate the biological activity of day 12 decidual supernatants in this assay, and similar results were obtained in all four experiments. Figure 3.9 shows the results of a representative experiment. IFN-γ was produced at a significant level when only control medium was added to the culture. Addition of anti-IL-10 antibody had no effect on IFN-γ production. Thus, IL-10 was not produced endogenously by IL-2 stimulated-spleen cells in this assay. On the other hand, day 12 decidual supernatant as well as recombinant IL-10 at a higher concentration inhibited the release of IFN-γ by IL-2-activated spleen cells. The inhibition was blocked by adding monoclonal anti-IL-10 antibody to the cultures. These results confirmed the presence of IL-10 in the decidual supernatants.

Interestingly, IFN-γ production by IL-2-stimulated spleen cells in the presence of diluted decidual cell supernatant (0.2-1.6%) was much higher than the medium control. This stimulation of IFN-γ production was partially blocked by anti-IL-10 antibody. In addition, IFN-γ production in the presence of decidual cell supernatant at a certain concentration range (0.2-25%) with anti-IL-10 antibody was also higher than the medium control. These results were obtained from all four different experiments, which excludes the possibility of an artefact. Possible explanations for this phenomenon are discussed in Chapter VI.

Thus, we confirmed by independent assays the presence of IL-3 and IL-10 in the decidual supernatants. Most important, decidual supernatant inhibited IL-2 stimulated IFN-γ production, which is one of the most characteristic biological functions of IL-10.

### C. Constitutive Production of IL-3, IL-4, IL-5, IL-10, and IFN-γ

We have found that the TH2-type cytokines IL-4, IL-5 and IL-10, the TH1 cytokine IFN-γ, as well as IL-3, were detectable in the supernatants of interface tissue cell cultures. Cytokines are generally produced in large amounts only by activated cells. Are these cytokine-secreting cells activated *in vivo* and remain activated in culture? Since the tissue culture medium contained 10% FCS, it is possible that the production of these cytokines was activated *in vitro*.

To examine this possibility, we determined the early time course of cytokine release into the culture supernatants as well as the intracellular cytokine content in the cell lysates. Since day 12 placenta can be separated into the fetal placenta and decidua, we can monitor the cytokine production by both maternal and fetal tissues.

Therefore we used pooled day 12 samples for the following investigation. Each experiment was repeated three times with similar results, and the results of representative experiments are shown here.

Cells from different tissues were washed, and cultured at $5x10^6$ cells/ml in RPMI+10% FCS for 15, 30, 60 and 120 minutes, before the supernatants were collected and the cells lysed in 1% Triton-X 100. Figure 3.10 shows that IL-3 and IL-10 were detectable in the supernatants within 15 minutes of culture, and IL-4, IL-5 and IFN-$\gamma$ within 60 minutes. They all reached a maximum level by 60 minutes.

The kinetics of cytokine appearance in the cell lysates are shown in Figure 3.11. Cell lysates of freshly isolated decidual and placental cells generally contained higher cytokine levels than those of cultured cells, and the latter declined as a function of time in culture. The fact that cytokines can be detected in the cell supernatants within 60 minutes as well as their presence in uncultured cells suggests that cytokine production is not induced in culture. Since the decidual and placental tissues were incubated with enzymes at $37^0$C for 45 minutes, the possibility of the cells being activated during this digestion period exists, but is not likely. Therefore, these cells are most likely producing these cytokines in vivo.

## D. Cytokine Production in Individual Mice.

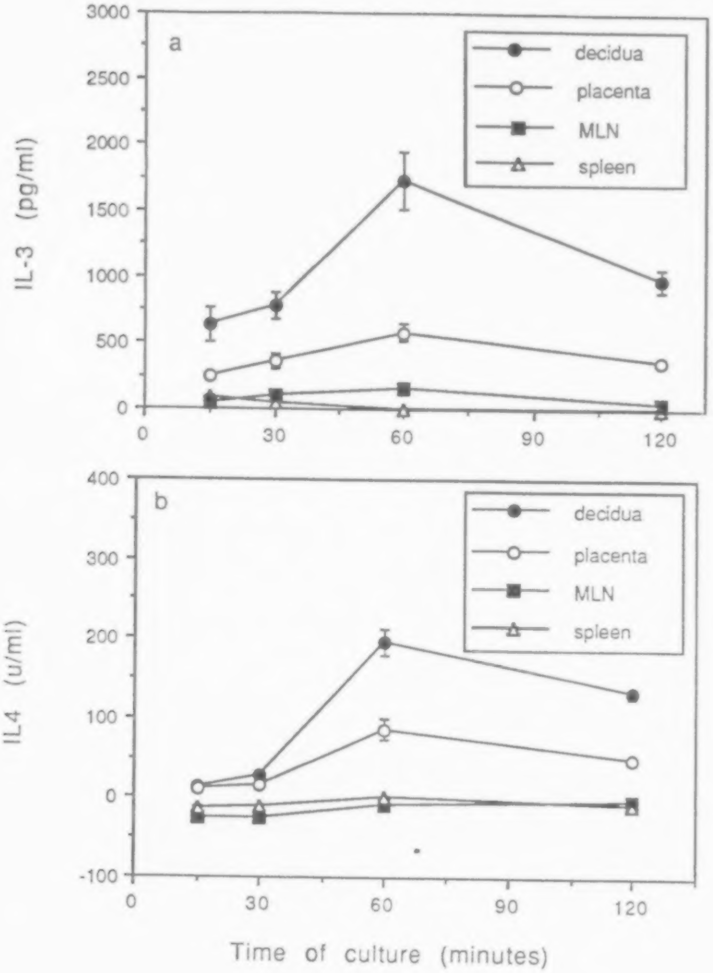
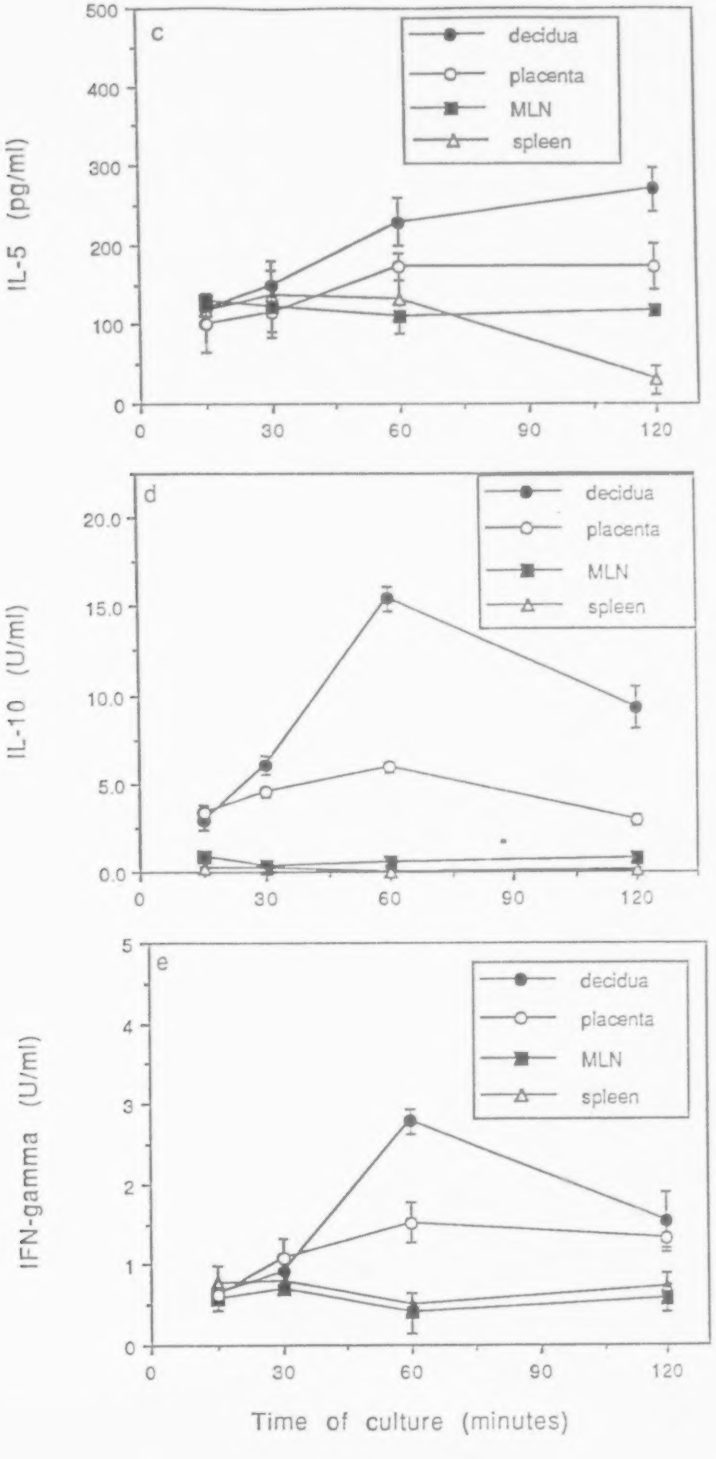


Figure 3.10. Kinetics of cytokine secretion from cells of day 12 pregnant mice.

Single cell suspensions were prepared from freshly isolated tissues from six Balb/c x Balb/c mice as described in the legend for Figure 3.1. Then samples from different mice were pooled and cultured at $5x10^6$ cells/ml in RPMI+10% FCS. Supernatants were collected at different time points and tested for IL-3 (Figure 3.10a), IL-4 (Figure 3.10b) IL-5 (Figure 3.10c), IL-10 (Figure 3.10d) and IFN-γ (Figure 3.10e) by ELISA. The results represent the mean and standard deviation of triplicate assays of the same samples. Each experiment was repeated three times with comparable results (Data not shown).


c
IL-5 (pg/ml)
500
400
300
200
100
0
0
30
60
90
120
decidua
placenta
MLN
spleen
d
IL-10 (U/ml)
20.0
15.0
10.0
5.0
0.0
0
30
60
90
120
decidua
placenta
MLN
spleen
e
IFN-gamma (U/ml)
5
4
3
2
1
0
0
30
60
90
120
decidua
placenta
MLN
spleen
Time of culture (minutes)

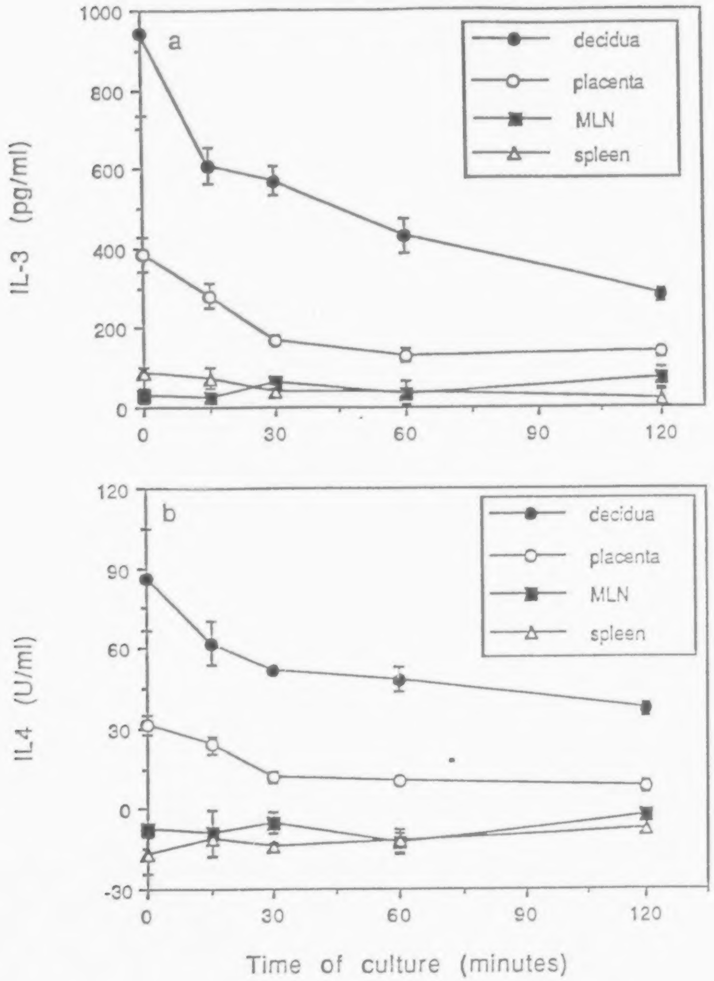


Figure 3.11. Cytokine levels in cell lysates.

The cells cultured as described in Figure 3.10 were lysed with 1% Triton X-100 at $5x10^6$ cells/ml after different periods of time in culture. The cell lysates were tested for cytokines by ELISAs. The results represent the mean and standard deviation of triplicate assays of the same samples. Each experiment was repeated three times with comparable results (Data not shown).

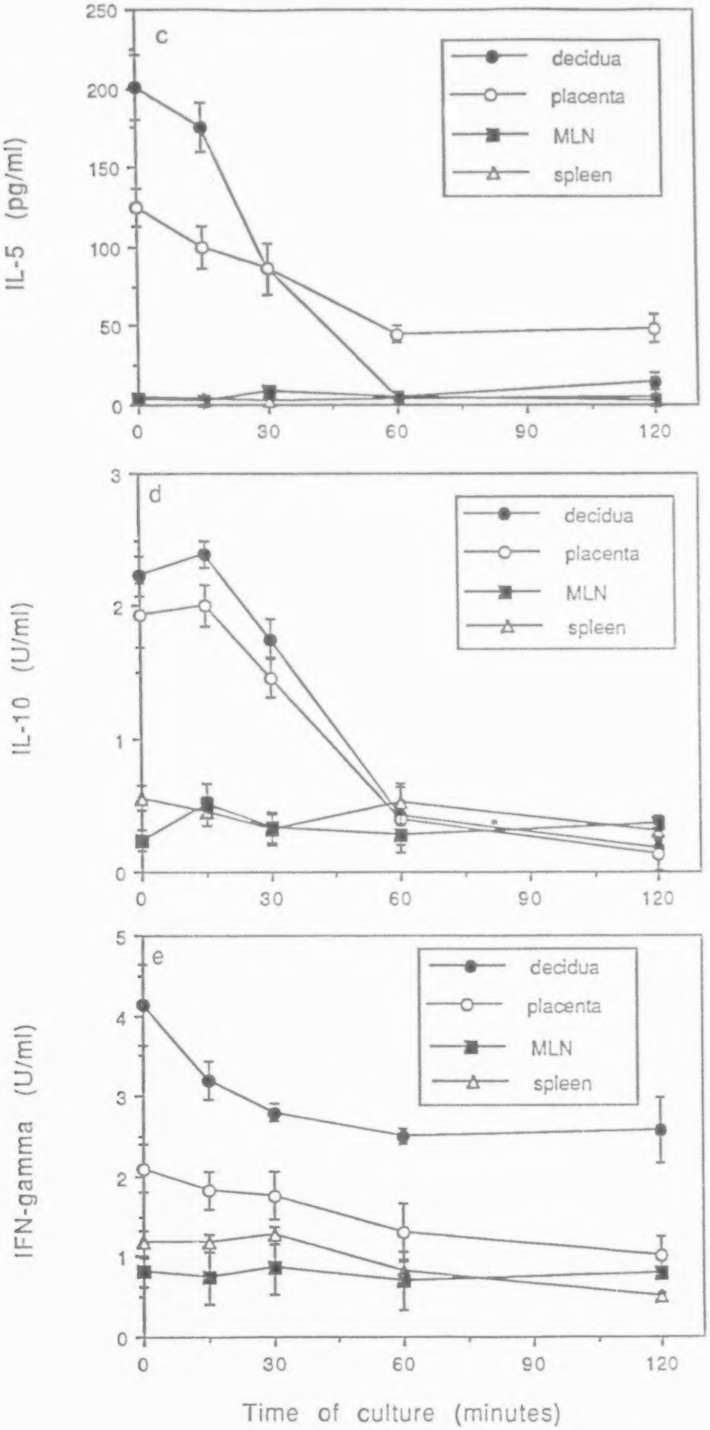

c
decidua
placenta
MLN
spleen
IL-5 (pg/ml)
250
200
150
100
50
0
0
30
60
90
120
d
decidua
placenta
MLN
spleen
IL-10 (U/ml)
3
2
1
0
0
30
60
90
120
e
decidua
placenta
MLN
spleen
IFN-gamma (U/ml)
5
4
3
2
1
0
0
30
60
90
120
Time of culture (minutes)

Table 3.1. Cytokine Levels in Supernatants of Decidual Cells Obtained from Individual BALB/c X BALB/c Day 12 Pregnant Mice.

| Mouse # | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-γ (U/ml) |
|---|---|---|---|---|---|
| 1 | 2612 ± 69[a] | 2119 ±248 | 2156 ±165 | 36.3 ± 2.0 | 4.1 ± 0.3 |
| 2 | 2473 ±143 | 2356 ±244 | 1675 ±234 | 37.6 ± 1.6 | 3.0 ± 0.3 |
| 3 | 618 ±147 | 1088 ±144 | 828 ± 64 | 16.9 ± 1.2 | 2.7 ± 0.2 |
| 4 | 1089 ± 21 | 1317 ±148 | 920 ± 41 | 21.6 ± 1.7 | 3.6 ± 0.2 |
| 5 | 1170 ±132 | 867 ± 82 | 1063 ±103 | 33.2 ± 1.8 | 1.4 ± 0.25 |
| 6 | 755 ± 85 | 2203 ±176 | 935 ± 96 | 33.6 ± 2.8 | 1.7 ± 0.1 |
| Average | 1453 ±869[b] | 1658 ±642 | 1262 ±533 | 29.9 ± 8.5 | 2.7 ± 1.1 |

[a]. Mean and standard deviation of triplicate assays of the same sample. [b]. Mean and standard deviation of six samples.

Day 12 decidual cells were obtained as described in the legend for Figure 3.1. Cells from six different mice were cultured separately at 5x10$^6$ cells/ml in RPMI+10% FCS for one hour. The supernatants were collected and tested by ELISA.

Table 3.2 Cytokine Levels in Supernatants of Placental Cells Obtained from Individual BALB/c X BALB/c Day 12 Pregnant Mice

| Mouse # | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-γ (U/ml) |
|---|---|---|---|---|---|
| 1 | 2356 ±254[a] | 724 ± 72 | 278 ± 85 | 7.2 ± 1.3 | <0.6[c] |
| 2 | 2636 ± 78 | 875 ± 41 | 227 ± 42 | 9.1 ± 2.4 | <0.6 |
| 3 | 2103 ±149 | 1061 ± 98 | 343 ± 24 | 7.8 ± 1.2 | <0.6 |
| 4 | 1039 ±164 | 507 ± 84 | 158 ± 25 | 4.4 ± 0.8 | <0.6 |
| 5 | 822 ± 54 | 668 ± 52 | 318 ± 73 | 4.3 ± 0.8 | <0.6 |
| 6 | 2058 ±223 | 676 ± 78 | 554 ± 8 | 9.6 ± 1.4 | <0.6 |
| Average | 1836 ±734[b] | 752 ±191 | 313 ±135 | 7.1 ± 2.3 | <0.6 |

*a*. Mean and standard deviation of triplicate assays of the same sample. b. Mean and standard deviation of six samples. c. Below detectability.

Placental cells from six day 12 pregnant mice were cultured and the supernatants were tested separately as described in the legend for Table 3.1.

Table 3.3. Cytokine Levels in the Supernatants of Con A-Stimulated Spleen Cells from Non-pregnant BALB/c Mice

| Mouse # | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-γ (U/ml) |
|---|---|---|---|---|---|
| 1 | <125[c] | 222 ± 28.2[a] | <300[c] | 2.3 ± 0.1 | 68.7 ± 2.5 |
| 2 | <125 | 220 ± 6.4 | <300 | 1.1 ± 0.2 | 57.4 ± 3.8 |
| 3 | <125 | 155 ± 1.8 | <300 | 1.5 ± 0.2 | 73.0 ± 1.9 |
| 4 | <125 | 162 ± 20 | <300 | 1.1 ± 0.1 | 53.1 ± 5.1 |
| 5 | <125 | 148 ± 12 | <300 | 1.6 ± 0.1 | 53.1 ± 2.4 |
| 6 | <125 | 195 ± 7.9 | <300 | 1.3 ± 0.2 | 65.8 ± 0.4 |
| average | <125 | 184 ± 33[b] | <300 | 1.5 ± 0.5 | 66.1 ± 9.5 |

[a]. Mean and standard deviation of triplicate assays of the same sample. [b]. Mean and standard deviation of six samples. [c]. Below detectability.

Spleen cells were collected from six different non-pregnant BALB/c mice. Cells from different mice were washed with PBS three times and cultured separately with 5 μg/ml Con A in RPMI+10% FCS for 24 hours. The supernatants were collected and tested by ELISA. This experiment was repeated three times with comparable results (data not shown).

Up to this point all the experiments were done using pooled samples. Thus the production of these cytokines at the maternal-fetal interface is either directly associated with pregnancy or simply an occasional maternal response to some unknown stimulus in some mice, and therefore not expected to occur in all individuals contributing to the population pool.

In order to exclude the latter possibility, day 12 decidual and placental tissues were collected from individual BALB/c X BALB/c pregnant mice that were impregnated on different days and kept in different cages. Single-cell suspension of different tissues were cultured in RPMI+10% FCS for 1 hour before the supernatants were collected. All the samples were then tested by ELISA.

Table 3.1 shows that IL-3, IL-4, IL-5, IL-10, and IFN-γ were detectable in individual decidual cell supernatants, with variation in the amount produced by each mouse. IL-3, IL-4, IL-5 and IL-10 also were detected in placental cell supernatants (Table 3.2). In contrast, IFN-γ was not present in the placental cell supernatants (Table 3.2). Thus IL-3, IL-4, IL-5, IL-10 and IFN-γ were consistently found to be released by tissues at the maternal fetal interface in all individuals tested. This indicates that these cytokines are produced consistently at the interface.

### E. Comparison of Constitutive Interface Cytokine Release with that by Con A-Stimulated Normal Spleen Cells.

The cytokine profile of day 12 placental cells, which consists of IL-3, IL-4, IL-5, IL-10, but not IFN-γ, is a typical TH2 cytokine profile. In contrast, decidual cells produce the TH2 cytokines IL-4, IL-5, and IL-10, as well as IL-3 and the TH1 cytokine IFN-γ.

Is the cytokine profile of decidual cells biased towards TH2? To answer this question, we had to assess the relative amounts of the TH2 versus TH1 cytokines secreted by tissues at the maternal-fetal interface. However one cannot directly compare the unit values of different cytokines because they are compared to different standards, and thus bear no relation to each other. We therefore compared the TH2/TH1 cytokine ratios of decidual cell supernatants with those detected following Con A-stimulation of normal spleen cells from non-pregnant mice. Table 3.3 shows that Con A-stimulated spleen cells produced IL-4, IL-10, and IFN-γ, but not IL-3 and IL-5.

The overall TH2/TH1 cytokine ratios were very different between Con A-stimulated spleen cells and decidual cells. In decidual cell supernatants, the IL10/IFNγ ratio was 3:1 on day 6 and 10:1 on day 12. In contrast, this ratio was 1:45 in the spleen cell supernatants. Likewise the IL4/IFNγ ratios on day 6 and 12 decidual supernatants were 6:1 and 600:1 respectively, whereas this ratio was 3:1 in the spleen cell supernatants. Thus cytokine production at the maternal-fetal interface is biased towards the TH2 pattern.

## F. Summary.

We have demonstrated that TH2-type cytokines IL-4, IL-5, and IL-10, along with IL-3 are constitutively produced at the maternal-fetal interface throughout the normal mouse pregnancy. IFN-γ is released at the interface only at earlier stages of pregnancy. The TH2/TH1 cytokine ratios at the interface are much higher than those generated by Con A-stimulated spleen cells from non-pregnant mice. None of these cytokines are spontaneously produced by unstimulated spleen cells and lymph node cells.

# IV. RESULTS: DETERMINATION OF THE CELLULAR SOURCES OF TH2-TYPE CYTOKINES AT THE MATERNAL-FETAL INTERFACE

As described in the Chapter III, the TH2-type cytokines IL-4, IL-5, and IL-10, along with IL-3 were found to be produced by interface tissues throughout pregnancy. IFN-$\gamma$ also was produced during the early stages of pregnancy. Next, we examined the nature of the cells that secrete these cytokines. Since IL-10 plays a prominent role in the inhibition of fetus-damaging TH1 cytokines, we emphasized the localization of IL-10 secreting cells at the interface.

## A. Cytokine Production by Spleen and Lymph Node Cells.

We firstly examined the cytokine secretion potential of lymphocytes from the spleen, MLN, and PALN during pregnancy. Since the T cell is one of the candidates for the production of the TH2-type cytokines mentioned above, the potential of cytokine production by T cells was examined following stimulation by Con A, a T cell mitogen. The spleen, MLN, and PALN were removed from individual day 12 pregnant BALB/c X BALB/c mice. From these tissues, single cells were prepared, washed and cultured in RPMI+10% FCS with or without 5 ug/ml Con A. Samples from different mice were cultured separately. Supernatants of 24 hour cultures were collected and tested via the cytokine ELISA. The means and standard deviations of the cytokine levels are listed in Table 4.1. When Con A was not present in theculture, none of the spleen, MLN,

Table 4.1. Cytokine Levels in the Supernatants of Cultured Cells from Day 12 Pregnant Mice with or without the Presence of Con A

| Cell | Con A | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-$\gamma$ (U/ml) |
|---|---|---|---|---|---|---|
| Spleen | + | <125[a] | <37.5 | <300 | <0.6 | 2.6 + 0.4[b] |
| (n=8)[c] | - | <125 | <37.5 | <300 | <0.6 | <0.6 |
| MLN | + | <125 | <37.5 | <300 | <0.6 | <0.6 |
| (n=5) | - | <125 | <37.5 | <300 | <0.6 | <0.6 |
| PALN | + | <125 | <37.5 | <300 | <0.6 | <0.6 |
| (n=5) | - | <125 | <37.5 | <300 | <0.6 | <0.6 |

[a]. Below detectability. [b]. Mean and standard deviation of replicate samples. [c]. Number of animals used.

Cells from the spleen, MLN, and PALN were obtained from different day 12 pregnant BALB/c X BALB/c mice. The cells were washed three times with PBS and cultured separately in RPMI+10% FCS with or without 5 μg/ml Con A. Supernatants were collected after 24 hours of culturing and tested by ELISA. Comparable results were obtained in two other experiments.

and PALN supernatants contained any detectable IL-3, IL-4, IL-5, IL-10, or IFN-γ. In the Con A-stimulated spleen cell supernatants, only a low level of IFN-γ was detected. None of the cytokines was detected in Con A-stimulated MLN and PALN cell supernatants. The same results were obtained from other two experiments.

These results indicate that lymphocytes in different peripheral lymphoid organs, including the uterine draining PALN, do not spontaneously secrete TH2-type cytokines. Furthermore, even when stimulated with Con A, which is a T cell mitogen, they do not produce significant levels of TH2-type cytokines. Therefore, these lymphocytes appear not to be involved in the TH2-like cytokine production during gestation, at least in these experimental conditions.

### B. Cytokine Production at the Maternal-fetal Interface in Immunodeficient Mice.

Although spleen and lymph node cells do not produce significant amounts of TH2 cytokines in culture, the possibility is not excluded that lymphocytes are activated and secrete cytokines within interface tissues. We next examined whether lymphocytes at the interface tissues are involved in TH2 cytokine production by two different approaches.

First, we isolated different cell subsets from decidual cells according to their surface markers by panning and magnetic bead separation. This procedure proved to be technically difficult. Cells had to be cultured at least 3-4 hours in the medium containing a low concentration of FCS before they were separated. Even unseparated cells did not secrete any detectable cytokine into the culture after these treatments (data not shown). Thus, this approach was not pursued further.

Second, we studied immunodeficient mice. The severe combined immunodeficiency (SCID) mouse carries a spontaneously arising autosomal recessive mutation. This strain was first observed to have severe combined immunodeficiency by Bosma (Bosma et al, 1983), and then was demonstrated to carry a defective VDJ recombinase system. Therefore, SCID mice have neither functional T cells nor B cells when they are young (Schuler et al, 1986).

The beige (bg) mouse has a mutation which selectively impairs NK cell function (Roder et al, 1979). A line of double mutant Scid/Scid·bg/bg mice was established by Croy and her colleagues (Croy and Chapeau, 1990). This strain of mice does not have functional T cells, B cells or NK cells. By studying these immunodeficient mice, we asked the question whether the "lymphocyte-depleted" interface tissues still make TH2-like cytokines.

Since the SCID and SCID·beige mice were available in Dr. Ann Croy's laboratory at University of Guelph, we collaborated with this group to carry out these experiments. In their laboratory, interface tissues were dissected out from day 6 and day 12 syngeneic pregnant SCID (scid/scid) and SCID·beige (scid/scid·bg/bg) mice. Then interface tissues of day 6 pregnant mice and interface cells of day 12 pregnant mice were processed and cultured according to the protocol provided by us, which was described in Chapter III. Supernatants were collected, frozen, and shipped to us. Then these samples were tested with cytokine ELISAs in our laboratory. BALB/c X BALB/c syngeneic pregnant mice and at a comparable stage of pregnancy from our colony at University of Alberta were used as normal control mice.

Table 4.2 shows the results of day 6 samples. Supernatants of decidual cells from both SCID and SCID·beige mice contained IL-3, IL-4, and IL-10, and without detectable IL-5 and IFN-γ. Supernatants of cultured embryos from these mice contained none of the cytokines examined. Similar results were obtained from day 12 samples shown in Table 4.3. Supernatants of decidual and placental cells from SCID and SCID·beige mice contained significant levels of IL-3, IL-4, and IL-10, with lower levels of IL-3 and IL-10 than those from BALB/c mice. There was no detectable IL-5 or IFN-γ in the these samples.

The levels of IL-3 and IL-10 produced in SCID and SCID·beige mice were lower than those in control BALB/c mice at both day 6 and day 12. The significance of these observations is discussed in detail in Chapter VI.

These results indicate that IL-3, IL-4 and IL-10 are produced in the absence of lymphocytes at the interface. IL-5 and IFN-γ were produced in BALB/c control mice, not in immunodecifient mice. However, one must bear in mind that the control mice were not from the same colony as the SCID and the SCID·beige mice. We therefore cannot exclude the possibility that the lack of IL-5 and IFN-γ

Table 4.2. Cytokine Levels in the Supernatants of Cells from Day 6 Pregnant Mice

| Mice | Cells | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-γ (U/ml) |
|---|---|---|---|---|---|---|
| SCID | Decidua | 365 ± 37[a] | 264 ± 60 | <300[b] | 1.9±0.8 | <0.6 |
| (n=6)[c] | Embryo | <125 | <37.5 | <300 | <0.6 | <0.6 |
| SCID-BEIGE | Decidua | 925 ± 196.1 | 296 ± 35 | <300 | 6.7±3.7 | <0.6 |
| (n=6) | Embryo | <125 | <37.5 | <300 | <0.6 | <0.6 |
| BALB/c<br>(n=6) | Conceptus | 18600 ±3500 | 375 ±156 | 365 ± 65 | 26.5 ±5.9 | 7.4 ± 3.2 |

[a] Mean and standard deviation of replicate samples. [b] Below detectability. [c] Number of animals used.

Day 6 tissues were dissected out and cultured separately at 10 tissues / ml in RPMI+10% FCS for six hours. Supernatants were then collected and tested by ELISA.

Table 4.3. Cytokine Levels in the Supernatants of Cells from Day 12 Pregnant Mice

| Mice | Cells | IL-3 (pg/ml) | IL-4 (U/ml) | IL-5 (pg/ml) | IL-10 (U/ml) | IFN-γ (U/ml) |
|---|---|---|---|---|---|---|
| SCID | Decidua | 458 ±411[a] | 670 ± 96 | <300[b] | 7.3 ± 1.7 | <0.6 |
| (n=6)[c] | Placenta | 4891 ±905 | 2282 ±465 | <300 | 10.1 ± 3.2 | <0.6 |
| SCID·BEIGE | Decidua | 651 ±278 | 351 ± 40 | <300 | 9.6 ± 2.9 | <0.6 |
| (n=6) | Placenta | 755 ±117 | 321 ± 70 | <300 | 13.9 ± 6.5 | <0.6 |
| BALB/c | Decidua | 1453 ±869 | 1658 ±642 | 1262 ±533 | 29.9 ± 8.5 | 2.7±1.1 |
| (n=6) | Placenta | 1836 ±734 | 752 ±191 | 313 ±135 | 7.1 ± 2.3 | <0.6 |

[a] Mean and standard deviation of replicate samples. [b] Below detectability. [c] Number of animals used.

Supernatants of cultured day 12 decidual and placental cells were collected as described in the legends for Tables 3.1 and 3.2, and tested by ELISA.

production in the immunodecifient mice was caused by different housing or enviromental antigens.

## C. Localization of Cells Expressing IL-10 mRNA at the Maternal-fetal Interface.

We next examined the location of these cytokine-secreting cells at the interface tissue. Our first approach was immunohistochemical staining. The cryostat tissue sections of placenta from pregnant mice were fixed and stained with different antibodies against IL-3, IL-4, IL-5, IL-10, and IFN-$\gamma$. We did not detect any positive signal on tissue sections. This indicated that the cytokine expression by the tissues was too low to be detected by this method. Therefore, this approach was not continued.

Our next approach was *in situ* hybridization. IL-10 inhibits TH1 cytokine production, leading to decreased cellular immunity. IL-10 was found to be produced constitutively at the protein level by interface tissues (Chapter III), which may provide an explanation for suppressed cellular immunity during pregnancy. We therefore decided to investigate the expression of IL-10 mRNA by the interface tissues. $^{35}$S-labeled IL-10 RNA probes were generated from Bluescript SK- containing the 582 bp Xba I-Not I fragment of the IL-10 cDNA (pIL-10$\delta$). The size of insert was confirmed by double digestion of the plasmid. As shown in Figure 4.1 (lane 3), a band of about 580 bp was produced by digesting plasmid with both Xba I and Not I. The purified plasmid was linearized with Not I (Figure 4.1, Lane 1) and transcribed with T7 RNA polymerase to generate the

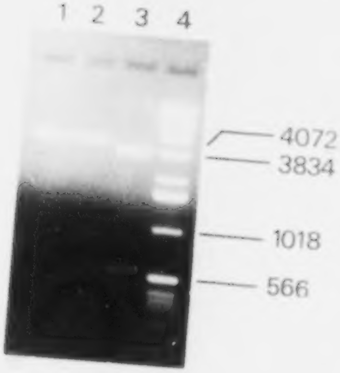
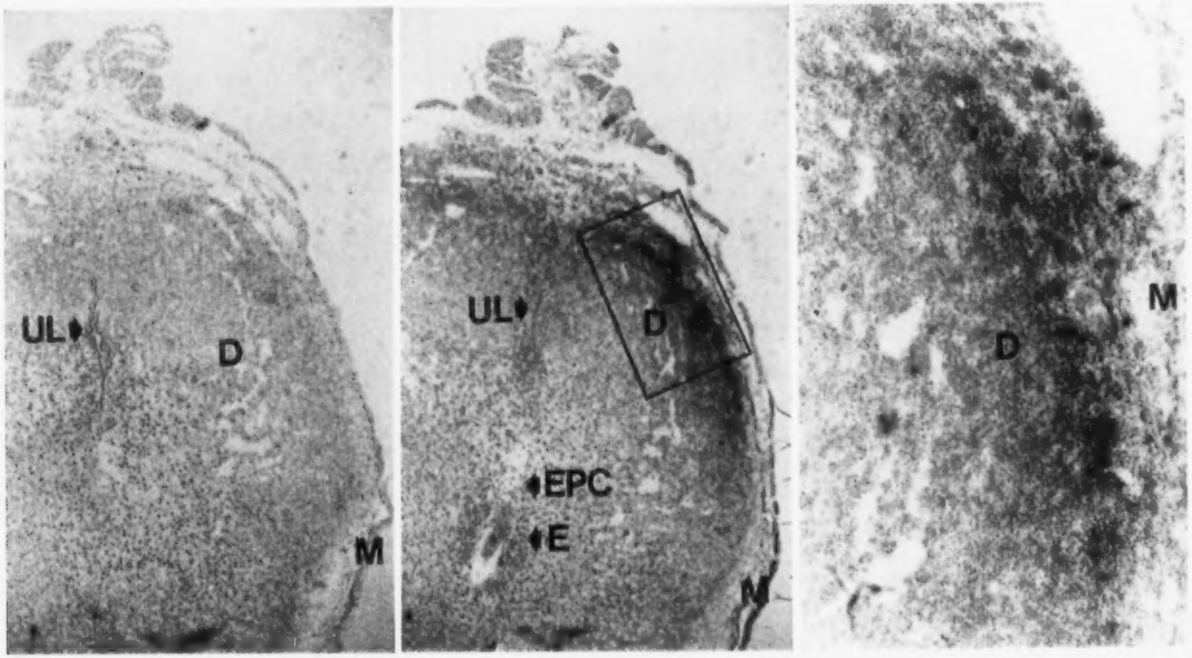


Figure 4.1. Restiction analysis of vector Bluescript $SK^-$ containing pIL-10δ.

Lane 1: Xba I digestion of the vector. Lane 2: Not I digestion of the vector. Lane 3: Xba I/Not I double digestion of the vector. Lane 4: 1 kb DNA ladder.

Figure 4.2. Autoradiograph of *in situ* hybridization for IL-10 mRNA expression in a tissue section of the uterus from a day 6 BALB/c X BALB/c mouse.

D: decidua; EPC: ectoplacental core; E: embryo; M: myometrium; UL: uterine lumen. The tissue sections were exposed to an anti-sense probe (b and c) and a control sense probe (a), and then counterstained with hemotoxylin. Cells that express IL-10 mRNA (dark reaction product) can be seen to be localized to the decidual layer (b and c).


UL
D
M
UL
D
EPC
E
M
D
M

a. Sense (X50)    b. Anti-sense (X50)    c. Anti-sense (X200) sense probe, or with Xba I (Figure 4.1, Lane 2) and transcribed with T3 RNA polymerase to generate the anti-sense probe.

Both anti-sense and sense probes were hybridized with day 6 placental tissue sections. Three experiments were done separately at different times, using three different pregnant females, and the results of a representative experiment are shown here. Figure 4.2b shows a cross section of the uterus at the implantation site on day 6. The embryonic cylinder can be seen with its ectoplacental cone (EPC) towards the uterine lumen. It is centrally located and occupies a very small region compared to that of the surrounding maternal decidua at this stage of development. Cells expressing IL-10 mRNA were localized within the outer zone of decidua on the mesometrial side. The morphology of these cells was not sufficiently well-defined to identify the cell type expressing IL-10 mRNA. Signal was not detected within the embryonic tissues including the EPC (Figure 4.2b and 4.2c). Hybridization with the sense probe as control was negative (Figure 4.2a).

## D. Summary.

Lymphocytes in the spleen and lymph nodes are unlikely to be involved in TH-2 cytokine production during pregnancy. IL-3, IL-4 and IL-10 are produced in the absence of lymphocytes at the maternal-fetal interface. The data on IL-5 and IFN-$\gamma$ are not conclusive due to the lack of a colony-matched control. Cells with abundant IL-10 mRNA are located within the maternal decidua. The type of cell(s) producing IL-10 has not yet been identified by the method employed here.

# V. RESULTS: DETERMINATION OF CYTOKINE PRODUCTION AT THE MATERNAL-FETAL INTERFACE IN FETAL RESORPTION-PRONE MICE

We have found that the TH2 cytokines IL-4, IL-5, IL-10, as well as IL-3 and, at low levels the TH1 cytokine IFN-γ are produced at the maternal-fetal interface in normal pregnant mice. IL-3, IL-4 and IL-10 are also produced by the interface tissues in immunodeficient mice. Next we examined functional correlates of these cytokines at the maternal-fetal interface.

Our approach was to study CBA X DBA/2 mice, which have been extensively studied for their high spontaneous fetal resorption rates (Clark et al, 1980). Different mechanisms have been postulated to explain the high fetal resorption rate in these mice. Among other mechanisms, cytokines were shown to influence the resorption rate (Chaouat et al, 1990). Injection of IL-3, GM-CSF led to decreased fetal resorption rates while injection of IL-2, IFN-γ or TNF-α had the opposite effect. Furthermore, the production of TGF-β was reduced (Clark et al, 1991), and NK cell infiltration was increased at the interface in CBA X DBA/2 mice compared to matings of other strain combinations (Gendron and Baines, 1988).

Using this mouse abortion model, we examined the production of TH2 and TH1 cytokines by the interface tissues from these mice. We also treated CBA X DBA/2 mice with ovine trophoblast protein (oTP). oTP is known to have immunosuppressive properties. It suppresses T cell blastogenesis *in vitro* (Newton et al, 1989). It may also be involved in the inhibition of local immune responses during the peri-implantation period (Fillion et al, 1991). Since CMI has deleterious effects on the fetus, oTP may be beneficial for fetal survival by prevention of fetal resorption.

Unfortunately the CBA X DBA/2 mouse model has not been working well in our laboratory for unknown reasons. Because the fetal resorption rate in these mice is influenced by environmental factors (Hamilton and Hamilton, 1987), it is possible that the environment at our animal facility does not provide the conditions required for the model. For instance, the variations of fetal resorption rates at different mouse colonies may be explained by the cytokines produced in vivo which are stimulated by environmental antigens, which can be influenced by background immune responses in different mouse colonies (Street et al, 1990). There is evidence to suggest that our mouse colony may be biased towards TH2 responsiveness, compared to those in an animal colony at DNAX, Palo Alto, CA (Mosmann, T., personal communication). Since the CBA X DBA/2 mouse model has been working well in Dr. G. Chaouat's laboratory at Clamart, France, we therefore collaborated with him. The experiments presented in this chapter were designed by our group, done partially in Dr. Chaouat's laboratory, and completed in our laboratory.

## A. Fetal Resorption Rates in Normal Mice and Fetal Resorption-prone Mice with or without Treatment.

These experiments were done in Dr. Chaouat's laboratory. Three groups of mice were used: CBA X DBA/2 mice, CBA X DBA/2 mice which had been injected with a single dose of oTP (0.6 μg i.p.) on day 6, and control CBA X BALB/c mice. There were 10 mice in each group. The fetal resorption rates were examined on day 12 of pregnancy.

As shown in Figure 5.1, the fetal resorption rate of control CBA X BALB/c mice was below 10%. In contrast, CBA X DBA/2 mice had an average resorption rate of 40%. The oTP injected CBA X DBA/2 mice had a fetal resorption rate below 10%. Thus, a single dose of oTP strongly prevented fetal resorption in CBA X DBA/2 mice.

### B. Cytokine Production by Cultured Interface Tissues from Normal, Fetal Resorption-Prone (FRP), and oTP Treated FRP Mice

Because of the high fetal resorption rates, there were fewer intact conceptuses in CBA X DBA/2 mice. Because of limited amounts of interface tissue from day 12 pregnant mice, tissues instead of cells were directly cultured. Decidual and placental tissues from each conceptus were dissected and cultured separately in 1 ml RPMI+10% FCS. After 24 hours, the culture supernatants were collected, lyophilized, and shipped to us. Then lyophilized samples were reconstituted with water and tested for cytokines by ELISA in our laboratory.

As shown in figure 5.2, significant levels of IL-3, IL-4, and IL-10 were detected in the supernatants of cultured decidual and placental tissues from normal CBA X BALB/c mice. No significant

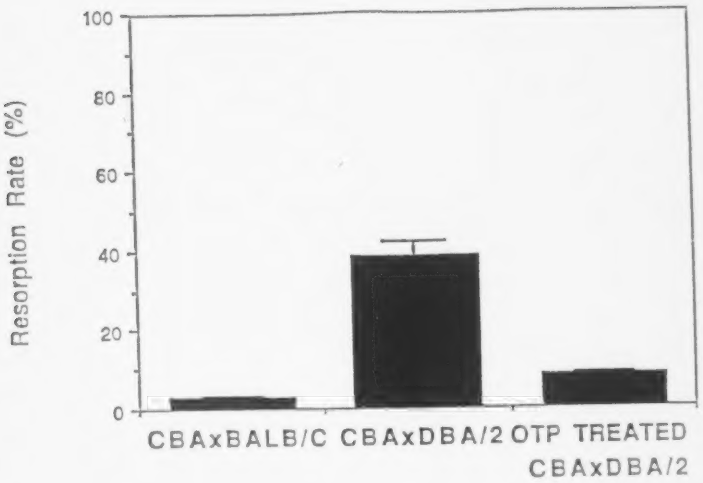
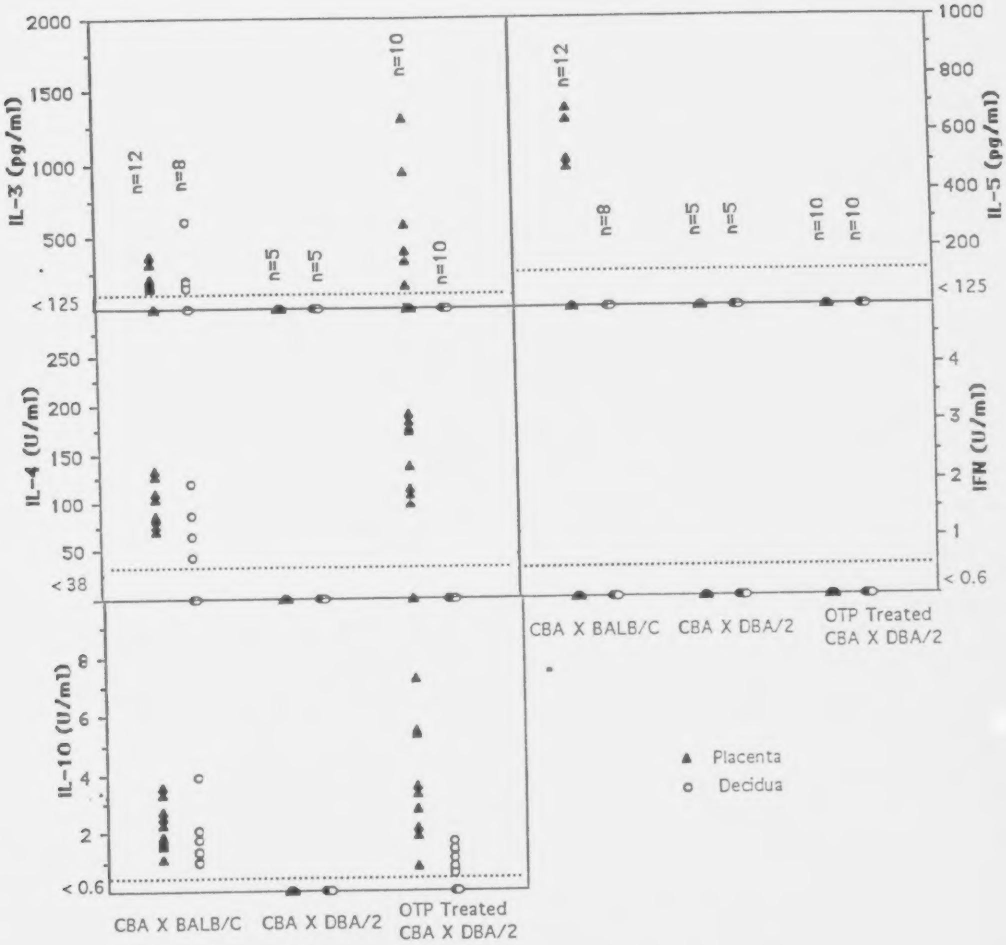


Figure 5.1. Fetal resorption rates as a function of strain combination and OTP ingestion.

Fetal resorption rates were examined on day 12 of pregnancy. 0.6 μg recombinant oTP was injected i.p. on day 5 of pregnancy. Ten mice were used in each strain combination. Data represent the mean and standard deviation of ten replicates. The fetal resorption rates of CBA x BALB/c and oTP treated CBA x DBA/2 mice were significantly lower than those of untreated CBA x DBA/2 mice ($p<0.001$).

Figure 5.2. Cytokine levels in the supernatants of the cultured interface tissues from normal, abortion-prone and abortion-prevented mice.

Interface tissues were taken from the mice that were used in the previous experiment shown in Figure 5.1. Decidual and placental tissues from one conceptus were cultured in one mililiter RPMI+10% FCS for 24 hours. Culture supernatants were tested by cytokine ELISA. Each point in the figure represents the sample from each conceptus. Dotted lines represent the detectability of each assay, and the points below these lines were not detectable by the assays.


IL-3 (pg/ml)
2000
1500
1000
500
< 125
n=12
n=8
n=5
n=5
n=10
n=10
IL-5 (pg/ml)
1000
800
600
400
200
< 125
n=12
n=8
n=5
n=5
n=10
n=10
IL-4 (U/ml)
250
200
150
100
50
< 38
IFN (U/ml)
4
3
2
1
< 0.6
IL-10 (U/ml)
8
6
4
2
< 0.6
CBA X BALB/C
CBA X DBA/2
OTP Treated CBA X DBA/2
CBA X BALB/C
CBA X DBA/2
OTP Treated CBA X DBA/2
▲ Placenta
○ Decidua

levels of IL-5 and IFN-γ were detected in these samples. In contrast, none of the cytokines tested were detected in the supernatants of cultured deciduas and placentas from CBA X DBA/2 mice. The placental supernatants from the oTP-treated CBA X DBA/2 mice contained significant levels of IL-3, IL-4, and IL-10 while only IL-10 was detected in the decidual supernatants. None of the other cytokines were detected in these samples. These initial results are in the process of being repeated and expanded.

### C. Summary.

A single dose of oTP can prevent frequent fetal resorption. The production of the TH2 cytokines IL-4, and IL-10, as well as IL-3, by cultured interface tissues is inversely correlated with fetal resorption in the strains of mice tested, while IL-5 and IFN-γ show no clear relation to this process.

# VI. DISCUSSION

The mouse placenta is a naturally occuring allograft in which the embryonically derived trophoblast expressing a low level of paternal MHC antigens comes directly in contact with maternal tissue in the decidua and blood stream (Hunt and Orr, 1988; Philpott et al, 1988; Hunziker and Wegmann, 1986). The maternal immune system recognizes the presence of this semi-allogeneic tissue (Van der Werf, 1971; Bell and Billington, 1980; Lala et al, 1986) and influences reproductive outcome both positively and negatively. Maternal T cells seem to enhance reproductive performance (Athanassakis et al, 1990), whereas NK cells are involved in the induction of fetal demise (Gendron and Baines, 1988; Kinsky et al, 1990). On the other hand, the maternal immune response is altered during gestation. Pregnancy is associated with a diminished ability of the host to resist infections by various intracellular pathogens (Bruce Chwatt, 1983; McGregor, 1984; Jensen et al, 1984; Koonin et al, 1989). Certain autoimmune diseases also change their course during pregnancy (Persellin, 1976; Varner, 1991). Thus, there is a bidirectional interaction between the maternal immune system and the reproductive system during pregnancy.

Cytokines seem to have an essential role in this bidirectional interaction. GM-CSF, IL-3 and CSF-1 promote the growth and the differentiation of trophoblast *in vitro* (Armstrong and Chaouat, 1989; Athanassakis et al, 1987), and GM-CSF and IL-3 enhance fetal survival *in vivo* (Chaouat et al, 1990). In contrast, IL-2, TNF-α and IFN-γ have deleterious effects on the fetus (Chaouat et al, 1990).

Various cytokines have been demonstrated to be produced by immunological and non-immunological components of both the fetus and the mother (Yelavarthi et al, 1991; Croy et al, 1991; Clark et al, 1991; Roberson et al, 1992; Stumpf et al, 1992; Bhatt et al, 1991; Kanzaki et al, 1991). However, our knowledge of cytokines released by various tissues is limited, especially at the maternal-fetal interface. The exact pathways of this bidirectional interaction are not clear.

The experiments described in this thesis were designed to further investigate the cytokine network at the maternal-fetal interface and its role in the interaction between the maternal immune system and reproductive tissues. The following discussion is organized into topics according to the questions addressed in the result sections (Chapters III-V).

## A. Constitutive Production of TH2-type Cytokines at the Maternal-fetal Interface during Normal Pregnancy.

The results presented in Chapter III indicate that a group of TH2-type cytokines, including IL-4, IL-5 and IL-10, along with IL-3, were synthesized by cells at the maternal-fetal interface in all three trimesters of pregnancy. IFN-$\gamma$ was also released by these cells, maximally at day 6 with declining levels thereafter.

In order to examine whether the cytokine profile of decidual cells is biased towards TH2, we had to assess the relative amounts of TH2 versus TH1 cytokines released in culture. We therefore compared the TH2/TH1 cytokine ratios with those of Con A-stimulated normal spleen cells. Cytokine secretion of lymphocytes can be readily induced in vitro by mitogen. The T cell mitogen Con A is often used to induce T cell-based cytokine production, and the cytokine profile of Con A-stimulated spleen cells is well characterized. The Con A induced spleen cytokine profile can be taken as an indication of overall TH1/TH2 ratios in the peripheral immune system, and therefore used as a basis for comparison with the ratios detected in the supernatants from the cultured interface tissues. According to this criterion, TH2/TH1 cytokine ratios were dramatically increased in supernatants from the cultured interface tissues compared with Con A-stimulated spleen cells from virgin mice. These results suggest that placental IL-10 progressively downregulates the local production of IFN-$\gamma$ during gestation. Support for this hypothesis was obtained by the ability of day 12 decidual cell supernatants to inhibit IL-2-induced release of IFN-$\gamma$ from normal spleen cells. Unstimulated spleen and lymph node cells from pregnant mice did not secrete detectable levels of the above cytokines.

The release of these cytokines from interface tissues is not due to *in vitro* stimulation by FCS in the culture, because substantial amounts of cytokines were detected in the supernatants within the first 15-60 minutes of culture, and freshly isolated cells lysed in the absence of tissue culture contain readily detectable levels. Since day 12 and day 18 interface tissues were digested with enzymes at $37^{0}$C for 45 minutes, the possibility cannot be excluded that these cells are activated during this digestion. But undigested day 6 conceptuses produced significant amounts of cytokines, which indicates cytokine secretion can exist without digestion. We thus regard it as unlikely that the cytokine secretion is activated by enzyme digestion, and therefore interface tissues most likely produce these cytokines in vivo. This conclusion is strongly supported by the in situ hybridization experiment discussed later.

The most intriguing finding in the current study is the constitutive release of IL-10 and IL-4 at the maternal-fetal interface. IL-10 selectively suppresses TH1-mediated cellular immunity. It does so by strongly inhibiting the production of inflammatory cytokines such as IFN-γ, TNF-α and IL-1 (Mosmann and Moore, 1991). IL-10 thus joins TGF-β2 and α-fetoprotein, among other substances, which play a potentially immunosuppressive role at the maternal-fetal interface (Clark et al, 1988). TGF-β2 inhibits IL-2 production at high concentrations and thus could be responsible for local immunosuppression (Clark et al, 1990). However, the most striking feature of the immunoregulation in pregnancy is the avoidance of cell-mediated immunity as well as complement fixing antibody production and enhancement of antibody production (Holland et al, 1984; Bell and Billington, 1980). The continuous presence of locally secreted IL-10 offers a plausible explanation for this phenomenon, because it is known for its ability to downregulate TH1 cytokines (Fiorentino et al, 1989). Most significantly, it inhibits the production of IFN-γ (Fiorentino et al, 1989). IFN-γ is a known abortifacient (Chaouat et al, 1990) and may act either by activating natural killer cells that can damage the trophoblast (Drake and Head, 1989) or by downregulating the production of trophoblast growth-promoting CSFs by the uterine epithelium (Robertson et al, 1992).

IL-4 has been known to be a key factor in determining the TH2 response against IFN-$\gamma$ mediated TH1 responses *in vivo*. Blocking of IL-4 activity *in vivo* with an anti-IL-4 antibody can shift a strong TH2 response to a TH1 response (Heinzel et al, 1989). Therefore, the production of IL-4 and a consequent increased ratio of IL4/IFN-$\gamma$ may be essential for maintaining the overall dominance of TH2 cytokines over TH1 cytokines under certain circumstances. IL-4 also regulates Ig isotype switching. It selectively induces the production of IgG1 and IgE (Coffman and Carty, 1986). Immunoglobulins of IgG1 isotype have been shown to be predominantly produced in allogeneic pregnant mice (Bell and Billington, 1980). Since these antibodies bind to the placenta and do not fix complement, which prevents possible complement-mediated cytotoxicity against placental cells, it has been postulated that these antibodies may play a protective role in the placenta (Voisin, 1980). The regulatory mechanism of IgG1 production has not been explained. The production of IL-4 at the interface may provide a possible explanation for this phenomenon.

Of the remaining TH2 cytokines, IL-5 is released by placental tissues. The presence of placental IL-6 has been described in human pregnancy, and IL-6 is released cultured uterine epithelial cells from pregnant mice (Nishino et al, 1990; Tabibzadeh et al, 1989; Robertson et al, 1992).

An additional finding of interest in the current study involves the constitutive secretion of IL-3. This cytokine was first implicated in reproductive events when it was shown to stimulate phagocytosis of trophoblast-like cells in the murine placenta (Athanassakis et al, 1990; Armstrong and Chaouat, 1989). It was subsequently shown to prevent spontaneous fetal resorption while augmenting fetal and placental size in CBA X DBA/2 pregnant mice (Chaouat et al, 1990). Thus, the presence of this lymphohematopoietic cytokine in tissues at the maternal-fetal interface may reflect its ability to promote growth and function of both the placenta and the fetus. The related cytokines, CSF-1 and GM-CSF, have also been described as being present at the interface and are secreted primarily by uterine epithelial cells under the influence of the hormones of pregnancy (Robertson et al, 1992; Arceci et al, 1989). IL-3 is considered to be primarily T cell-derived, but it is also known to be secreted by other cells (Frei et al, 1986; Gallo et al, 1991; Razin et al, 1991). The cellular source of placental IL-3 will be discussed in detail in the next section.

There is a interesting phenomenon that we observed in the IL-10 bioassay. IFN-γ production by IL-2-stimulated spleen cells was inhibited by day 12 decidual cell supernatants, which was in turn blocked by anti-IL-10 monoclonal antibody. This experiment confirmed our results from ELISA that decidual cell supernatants contain IL-10. It also indicated that decidual IL-10 is biologically active. In addition, in this experiment, we found that when decidual IL-10 was neutralized by anti-IL-10 antibody, the level of IFN-γ was higher than the medium control. The same result was not obtained when recombinant IL-10 was prevented from inhibiting IL-2-induced IFN-γ production by anti-IL-10 antibody. This excludes the possibility that there was endogenous IL-10 produced by IL-2-stimulated spleen cells in the assay. Therefore, decidual supernatants appear to contain another factor, which can stimulate IFN-γ production. This was consistent with our finding that diluted decidual supernatant (0.2-1.6%) stimulated instead of inhibited IFN-$\gamma$ production, which was also partially blocked by anti-IL-10 antibody. Thus, after decidual IL-10 is diluted out, this factor can stimulate IFN-$\gamma$ production, and the stimulation can be synergized by IL-10. We have no idea what this factor may be. It would be of great interest to further characterize this factor, but it was beyond the scope of this thesis.

In conclusion, we have described the constitutive production of a group of TH2-type cytokines at the maternal-fetal interface during normal murine pregnancy. Overall, the balance seems to be towards those cytokines which enhance humoral immunity while dampening cell-mediated immunity.

### B. Heterogenous Cellular Sources of TH2-type Cytokines

Since TH2-type cytokines are produced at the maternal-fetal interface during normal pregnancy, it is of great interest to examine where these cytokines originate. The results presented in Chapter IV show that peripheral lymphocytes did not produce any detectable TH2 cytokines in culture. IL-5 and IFN-$\gamma$ were not produced by "lymphocyte-depleted" fetoplacental units from SCID and SCID·beige mice. However, cultured interface tissues from SCID and SCID·beige mice still produce IL-3, IL-4, and IL-10.

The interpretation of experiments with SCID mice is always frought with concern about the problem of "leakiness". As a function of age and exposure to environment stimuli, some lymphoid progenitor cells in "leaky" SCID mice pass through the differentiation barrier to produce functional T and B cells (Bosma et al, 1988). To exclude "leaky" mice, only young mice (8-15 weeks) were used in this study. In addition, the mice were routinely assessed for the presence of Igs by ELISA, and only "non-leaky" SCID mice were used (see Chapter II for details). The fact that IL-3, IL-4, and IL-10 are produced by interface tissues in the absence of functional T cells, B cells and NK cells indicates that these three cytokines are produced by non-T, non-B, and non-NK cell type(s).

It was also observed that lesser amounts of IL-3 and IL-10 were secreted by cultured interface tissues from immunodeficient mice. This can be due to any of the following possibilities.

Firstly, since we did not test many different strains of mice in our study, we do not know the range of cytokine production by the interface tissues. The difference in the amount of cytokine production between immuno-deficient mice and BALB/c mice could be due to normal variation. Secondly, SCID and SCID·beige mice were kept in the colony at the University of Guelph, and control BALB/c mice were from the University of Alberta, and they were kept under very different conditions. SCID and SCID·beige mice were maintained in a pathogen-free environment, while control BALB/C mice were in ordinary housing. Since environmental antigens can influence the cytokine profile of mitogen-stimulated normal spleen cells (Street et al, 1990), it is possible that the differences in cytokine production are due to the different environments of the two colonies, e.g. lack of background stimuli in the pathogen-free house leads to low level of cytokine production. Thirdly, the difference could be due to the lack of functional lymphocytes in SCID and SCID·beige mice. IL-3 is produced by T cells, and IL-10 can be produced by T cells as well as B cells. Thus these cytokines may be directly produced by T cells, or their production may be under the regulatory control of T cells. A lessened production in the absence of T and B cells may therefore be due to a partial contribution by these cells to the overall cytokine profile.

More studies need to be done to clarify this matter. For example, cell separations, such as we have unsuccessfully attempted, might help to determine the contribution by various cell types. Nevertheless, we have established that TH2-type cytokines are produced by one or more non-lymphocytic cell type(s).

The maternal reproductive tract and placenta are known to be the sources of many cytokines. A variety of cytokines are produced by both lymphocytes and nonlymphohematopoietic cells, including maternal macrophages (Rambaldi et al, 1987; Flynn et al, 1982), uterine epithelial cells (Tamada et al, 1990; Roberson et al, 1992; Yelavarthi et al, 1991), metrial gland cells (Croy et al, 1991), and fetally derived placental trophoblast cells (Kanzaki et al, 1991; Tabibzadeh et al, 1989; Yelavarthi et al, 1991). Our determination that there are non-immune interface cellular source(s) of TH2-type cytokines, especially IL-10, expands our knowledge of the contribution from different cell types to the cytokine network at the maternal-fetal interface.

IL-10 can be produced by T cells (Fiorentino et al, 1989; Moore et al, 1990; MacNeil et al, 1990), B-1 cells (O'Garra et al, 1992), mast cells (Moore et al, 1990), keratinocytes (Enk and Katz, 1992), and macrophages (Fiorentino et al, 1991a). The fact that IL-10 is produced by fetoplacental units from immunodeficient SCID·beige mice suggests that IL-10 can be produced by non-lymphoid cell types.

The *in situ* hybridization studies also showed that IL-10 mRNA expressing cells were located at the outer layer of maternal decidua on day 6 tissue sections (Figure 4.2). Trophoblast (EPC) forms at the ectoplacental pole of the embryonic cylinder by about day 6 of gestation in mouse. Trophoblastic giant cells differentiate from EPC cells and invade into the surrounding maternal decidual tissue by day 6 - 6.5. Significant migration of these cells into the maternal decidua occurs at day 7 and later (Kaufman, 1992). There are no migrating trophoblastic giant cells within the outer decidual layer by day 6 of gestation. Therefore, the cells expressing IL-10 mRNA within this region are most likely maternal in origin.

Because the morphology of the frozen tissue sections was not well defined in any of the three experiments performed, we cannot identify the cell type that expresses IL-10 mRNA. The cell types that are in this region include stromal-type decidual cells, lymphocytes, macrophages, granulocytes and granulated metrial gland (GMG) cells.

Stromal-type decidual cells represent the most unique and distinctive cellular component in the decidual tissue. These cells are often large but variable in size. They are slightly vacuolated, occasionally binucleated, but mostly uninucleated cells of ovoid appearance. They have eccentric, round, or ovoid nuclei with uniformly dispersed chromatin containing one or more nucleoli. The cytoplasm is basophilic when stained with Giemsa (Lala et al, 1984).

They are immediate end products of proliferation and differentiation of endometrial stromal cells which may have been derived from blood-borne immigrant cells (Lala et al, 1984). Kearns and Lala (1982) reported that decidual cells within the decidual nodules of pseudopregnant mice are the ultimate descendants of bone marrow cells. It has thus been suggested that the majority of decidual cells are bone-marrow-derived. Decidual cells also share some surface markers of other bone-marrow-derived lineages. The majority of them express Thy-1 and the Fc receptor. Few or none of them express Lyt or I-A antigens (Lala et al, 1984). A unique surface marker of decidual cells, Dec-1, present on a major proportion of mature decidual cells, is also recognized by a monoclonal antibody (Lala et al, 1983). The function of decidual cells is multiple. Besides playing a putative nutritive role during pregnancy, they are believed to release chemical mediators and hormones (Lala et al, 1983). They also express mRNA of TNF-α (Yelavarthi et al, 1991) and the CSF-1 receptor (Arceci, et al, 1989), as detected by in situ hybridization. This suggests that they could be both the cellular source and target of some of the cytokines at the maternal-fetal interface. Our in situ hybridization study showed that the cells expressing IL-10 mRNA are localized at the outer zone of the decidua in a "patchy" pattern. These cells are very likely decidual cells. It is possible that only some decidual cells, under an unknown source of local regulation, produce IL-10. If this is true, it would be interesting to determine whether the IL-10 production by stromal cells is associated with the presence of the fetus. That can be done by examining whether IL-10 is produced by uterine endometrial stromal cells from non-pregnant and pseudopregnant mice.

IL-10 is known to be produced by macrophages. Maternal macrophages are abundant in the myometrium and metrial gland, but their number in the decidua is limited and their distribution is scattered throughout the decidua (Redline and Lu, 1989). Thus their distribution pattern is not coextensive with that of IL-10 mRNA.

Another possibility is that GMG cells express IL-10 because they have been shown to produce different cytokines, e.g. CSF-1, IL-1 and LIF (Croy et al, 1991). Although they are abundant in the area of the mesometrial triangle, they also occur in the decidua. A very small number of them has been noted in the decidua as early as day 3 of pregnancy (Smith, 1966). Following implantation, their number increases dramatically, and they are evenly distributed throughout the decidua (Stewart and Peel, 1978), which is different from the distribution of IL-10 mRNA-expressing cells.

Our in situ hybridization results are still preliminary. More experiments need to be done to confirm the results, especially the distribution and the morphology of the cells that expressing IL-10 mRNA, as well as the expression at various stages of gestation.

IL-3 and IL-4 are also produced by cells which are non-T, non-B, non-NK cells according to the results of our study. IL-4 is known to be produced by T cells (Isakson et al, 1982; Howard et al, 1982), mast cells (Brown et al, 1987; Seder et al, 1991; Paul, 1991) and basophils (Seder et al, 1991), and IL-3 can be produced by T cells (Schreier and Iscove, 1980; Kelso and Metcalf, 1985), keratinocytes (Gallo et al, 1991) and mast cells (Razin et al, 1991). In order to localize the cells that secrete IL-3 and IL-4, we stained the placental tissue sections with the monoclonal antibodies against IL-3 or IL-4, but no positive signal was detected in either case. It is thus necessary to use more sensitive methods, such as *in situ* hybridization, to determine the exact locations of the cells that secrete these cytokines. We already know that IL-3 can be produced by uterine epithelium (Robertson et al, unpublished data).

In contrast to the cytokines IL-3, IL-4, and IL-10, the cytokines IL-5 and IFN-γ are not produced by fetoplacental units from SCID mice which have T cell and B cell deficiency. IL-5 is generally considered to be a T-cell derived cytokine (Tominaga et al, 1988), whereas IFN-γ can be produced by T cells (Epstein et al, 1984) and NK cells (Trinchieri et al, 1984; Ortaldo et al, 1984). Because of the lack of control mice from the same colony, it cannot be ruled out that their absence is due to the influence of environment on cytokine production. Thus, colony-matched control needs to be done before we know whether IL-5 and IFN-γ production at the interface is lymphocyte-dependent.

In addition, lymphocytes from the uterus-draining PALN and distant MLN and spleen do not produce in culture any detectable IL-3, IL-4, IL-5, IL-10, or IFN-γ *in vitro*. When stimulated with Con A, which is routinely used to activate lymphocytes to produce cytokines, spleen cells produce only a low level of IFN-γ, and lymph node cells produce none of the cytokines examined. Therefore, lymphocytes in the peripheral lymphoid organs are unlikely to be involved in cytokine production .

As described previously, the maternal systemic immune response is biased during pregnancy. Pregnant females are more susceptible to infections caused by intracellular pathogens and are also more resistant to the induction of primary DTH and NK responses. On the other hand, PALN cells from pregnant mice produce a much higher plaque-forming cell response than do those of non-pregnant mice. Thus, the maternal systemic immune responses are directed away from CMI and towards antibody production.

Since there is no evidence that peripheral lymphocytes spontaneously produce TH2 cytokines *in situ*, TH2 cytokines produced locally at the maternal-fetal interface may be released into the maternal blood stream and influence the systemic maternal immune response. We thus sought the presence of cytokines in pregnancy serum.

Pregnancy serum has been known to be immunosuppressive, inhibiting mitogen-induced lymphocyte transformation (Wajner et al, 1985; Murgita, 1976) and allogeneic cytotoxicity (Smith, 1978). Although several factors, including progesterone (Siiteri et al, 1977; Clemens et al, 1979), $\alpha_2$-glycoprotein (Fernandez-Botran et al, 1986; Peck et al, 1978), and $\alpha$-fetoprotein (Hooper and Evans, 1989), have been reported to be immunosuppressive, none of them has been proven so unambiguously. Our attempts to detect IL-3, IL-4, IL-5, and IL-10 in the pregnancy serum have not been successful. Their levels in serum may be too low to be detected by the currently available assays. Alternatively, circulating immune cells may be influenced by TH2 cytokines at the placenta when they traverse this region.

The above results indicate that the cytokines can be produced by non-lymphoid cells. They contribute to a TH2-type cytokine network at the maternal-fetal interface.

### C. The role of TH2-type Cytokines in Fetal Survival

The CBA X DBA/2 mouse model of spontaneous abortion was first reported in 1980 (Clark et al, 1980). These mice have unusually high fetal resorption rates, ranging from 30% to 50%, while most strains have rates below 10% (Clark et al, 1980 and 1986). Since then this mouse model has been extensively studied to determine the underlying mechanisms.

Immunogenetic studies have demonstrated that the high resorption rate can be reduced to normal levels by immunizing the CBA X DBA/2 mice with white cells sharing paternal MHC haplotype prior to conception (Chaouat et al, 1983). Reduced resorption rates are also achieved by the footpad injection of the nonspecific immunopotentiator complete Freund's adjuvant (Toder et al, 1990). This suggests that the enhanced fetal resorption in these mice can be immunologically infuenced.

In our study, we examined the relationship of fetal resorption and TH2-type cytokine production at the maternal-fetal interface. Due to the high resorption rate, there were fewer intact conceptuses available in CBA X DBA/2 mice. We therefore cultured decidua or placenta from every conceptus in 1 ml of medium, in which the tissue density was lower than that of previous experiments. In this system, IL-3, IL-4 and IL-10 were still detectable in supernatants of cultured interface tissues from normal CBA X BALB/c mice, a result which was consistent with our previous results. As shown in Chapter III, these cytokines are constitutively produced by interface tissues throughout pregnancy. Therefore, although tissues were cultured for 24 hours in this experiment, the levels of cytokines detected in the supernatants are most likely a reflection of the amounts of cytokines produced in vivo. IL-5 and IFN-γ were not detectable in the same samples.

In contrast to the cytokine profile from CBA X BALB/c mice, supernatants of interface tissues from CBA X DBA/2 mice contained none of the cytokines examined, including IL-3, IL-4, IL-5, IL-10, and IFN-γ. Most importantly, IL-3, IL-4, and IL-10 were detected at significant levels in the supernatants of cultured interface tissues from CBA X DBA/2 mice in which the females were injected with oTP, which reduced fetal resorption rates. Thus, the lack of production of IL-3, IL-4, and IL-10 at the interface of CBA X DBA/2 mice is not due to a variation between mouse strain combinations. Instead it is associated with the high fetal resorption rates of these mice. Therefore, we have demonstrated an inverse correlation between the fetal resorption rate and the amounts of TH2 cytokines produced by cultured interface tissues, which most likely reflects the amounts of cytokines produced at the interface in vivo.

This is also supported by observations of Raghupathy's group (Raghupathy et al, personal communication). They examined the cytokine profiles of spleen cells that were activated *in vitro* by irradiated placental cells from mice of different strains. Spleen cells of the maternal strain (CBA) produced the TH2 cytokine IL-5 in response to CBA X BALB/c placental cells, whereas placental cells from CBA X DBA/2 matings induced predominantly the production of the TH1 cytokines TNF and IFN-γ by maternal lymphocytes. This result implies that normal placental cells can mediate a TH2 response, but placental cells from fetal resorption-prone mice induce a TH1 response.

However, we did not find any increased production of the TH1 cytokine IFN-γ by cultured interface tissues from CBA X DBA/2 mice. Because fewer conceptuses were available from these fetal resorption-prone mice, interface tissues were cultured at a very low density. Therefore, the possibility exists that the level of IFN-γ in the culture supernatant is too low to be detectable by ELISA. Thus, we cannot rule out the possibility that the production of IFN-γ by interface tissues from CBA X DBA/2 is increased compared to that from normal mice. On the other hand, in Raghpathy's experiment, IFN-γ was found in the supernatants of one way mixed spleen cell-trophoblast cultures. Thus, trophoblast cells from fetal resorption-prone mice may lose the ability to downregulate IFN-γ production by stimulated spleen cells. For example, one possibility is that decreased IL-10 production by trophoblast cells leads to increased IFN-γ by the spleen cells.

Based on previous studies, the role of TH2 cytokines in maintaining successful pregnancy can be explained by their inhibitory effects on TH1 cytokine production and NK activity as well as their beneficial effect on CSF cytokine production at the interface.

As previous stated, TH1 cytokines have deleterious effects on the placenta and the fetus. Injection of the TH1 cytokines IL-2, IFN-γ and TNF-α leads to increased fetal resorption rates. TNF-α inhibits DNA synthesis of rat trophoblast cell-lines (Hunt et al, 1989), proliferation of human choriocarcinoma cell lines (Berkowitz et al, 1988), as well as mouse embryonic development (Hill et al, 1987). It has also been shown recently that TNF-α can kill human trophoblast cells in culture (Yui, J., Wegmann, TG, Garcia-Lloret, M. and Guilbert, L. personal communication). IFN-γ has similar effects on trophoblast cells in vitro. It inhibits mouse embryo development and trophoblast outgrowth in vitro (Hill et al, 1987, Haimovici et al, 1991). It also inhibits the proliferation of human choriocarcinoma cell lines and induces the lysis of these cells in the presence of actinomycin-D (Fulop et al, 1992). Both TNF-α receptors (Hayakawa et al, 1991) and IFN-γ receptors (Calderon et al, 1988; Peyman and Hammond, 1992) are expressed by human trophoblast cells, which provides the molecular basis for the effects of these two cytokines on trophoblast cells. Most interestingly, the expression of the human IFN-γ receptor is constitutive, which is different from most cytokine receptors (Fulop et al, 1992). Therefore, the function of target cells expressing IFN-γ receptors is mainly regulated by the local concentration of IFN-γ. Because IL-10 inhibits the production of TH1 cytokines, especially IFN-γ, its presence locally at the interface may effectively prevent the production of large amounts of IFN-γ. Although utero-placental TNF-α production is not inhibited (Chen et al, 1992), it may be locally neutralized by soluble 55 kd human TNF-α receptors secreted by the syncytiotrophoblast (Austgulen et al, 1992a and 1992b). This balance is broken at term by a drop in production of the soluble TNF-α receptors (Austgulen et al, 1992a). A dramatically increased concentration of unbound TNF-α may play a role in the termination of pregnancy during parturition. Therefore, during normal pregnancy, amounts of bioactive TNF-α or IFN-γ could be regulated to be below the level that can cause deleterious effects on the trophoblast.

Maternal NK cells play an instrumental role in the etiology of spontaneous fetal resorption. In CBA X DBA/2 mice, NK cells are the predominant infiltrating cells in resorbing fetoplacental units (Gendron and Baines, 1988). They also appear in a significant proportion of fetoplacental units before fetal resorption is detectable (Gendron and Baines, 1988). This was confirmed by Kinsky and his colleagues (Kinsky et al, 1990). They reported that activation of NK cells in pregnant mice by the double stranded RNA poly-IC induces fetal resorption. This effect also is adoptively transferred with poly-IC treated spleen cells, but not if they are treated with anti-NK antibody. Therefore, NK cells can cause fetal resorption in mice of different strain combinations.

In addition, NK activity is decreased during normal pregnancy. The NK activity of peritoneal cells from pregnant mice that received *T. gondii* is significantly lower than that found in comparably treated virgin mice. Therefore, normal pregnancy is associated with an impaired ability to augment NK activity, which may be important for fetal survival (Luft and Remington, 1984). What inhibits NK activity during pregnancy? To answer this question, Chaouat and his colleagues examined immunological changes of fetal-placental units in fetal resorption-prone mice and found that placental cells from those mice have a decreased ability to suppress NK activity as compared with normal placental cells (Gendron et al, 1990). The molecular mechanism behind this phenomenon is not known, although it has been suggested that TGF-β production by the decidua is reduced in CBA X DBA/2 mice (Clark et al, 1991).

Our results suggest that TH2 cytokines might suppress NK cell activity in the fetal-placental unit during normal pregnancy. When the production of TH2 cytokine decreases in fetal resorption-prone mice, NK cells are activated, leading to fetal demise.

NK activity is regulated at least partially by TH1/TH2 cytokines. The TH1 cytokines IL-2, IFN-γ, and TNF are involved in different stages of NK activation and effector function (Phillips and Lanier, 1986), whereas the TH2 cytokines IL-4 and IL-10 suppress NK activity (Kawakami et al, 1989; Hsu et al, 1992).

IL-2 induces the generation of lymphokine-activated killer (LAK) cells and the production of IFN-γ and TNF-α from NK cells (Trinchieri et al, 1984; Phillips and Lanier, 1986; Ortaldo et al, 1986). IFN-γ and TNF-α are important for NK cell activation because antibodies against IFN-γ and TNF-α abrogate the IL-2-dependent induction of LAK cells (Karre et al, 1991). In addition, IL-2 and TNF-α activate trophoblast lytic LAK cells *in vitro* (Drake and Head, 1989; Clark, 1990). IL-2, IFN-γ and TNF-α are also abortifacient *in vivo* (Chaouat et al, 1990).

In contrast to TH1 cytokines, TH2 cytokines not only inhibit TH1 cytokine production but also have a direct inhibitory effect on NK activity. IL-4 inhibits IL-2-induced IFN-γ production by NK cells and prevents the generation of LAK cells (Spits et al, 1988; Kawakami et al, 1989; Hsu et al, 1992). IL-10 also inhibits IFN-γ production by IL-2 stimulated NK cells, but this inhibition process requires the presence of monocytes (Hsu et al, 1992), suggesting the presence of different and probably indirect inhibitory pathways. It is possible that this indirect pathway may be through the downregulation of IL-12.

IL-12 was firstly discovered to be produced by human B lymphoblastoid cells (Stern et al, 1990). It stimulates the proliferation of activated T cells and NK cells (Stern et al, 1990; Gately et al, 1991). It synergizes with IL-2 in the induction of LAK cells (Stern et al, 1990). It also induces IFN-γ production and enhances NK lytic activity by resting peripheral blood mononuclear cells (Chan et al, 1991). Mouse IL-12 was also cloned and shown to have a function similar to human IL-12 (Schoenhaut et al, 1992). Most importantly, it has recently been shown that IL-12 is produced by activated macrophages and its production may be important for the development of TH1 cells both in vitro (Hsieh et al, 1993) and in vivo (Heinzel et al, 1993). Moreover, IL-12 production by activated macrophages is downregulated by IL-10 (Hsieh et al, 1993; Mosmann, TR, personal communication). Therefore, by downregulating local IL-12 production by macrophages and/or other cells at the interface, IL-10 would inhibit the activation and IFN-γ production of NK cells.

The putative fetal resorption-preventing effect of TH2 cytokines can also be explained by their regulatory effects on the local production of CSF cytokines at the maternal-fetal interface. Cytokines of the CSF family are growth factors for placental trophoblast cells. GM-CSF enhances the proliferation and phagocytic function of placental cells *in vitro* (Athanassakis et al, 1987) and improves fetal survival *in vivo* (Chaouat et al, 1990). GM-CSF is produced by uterine epithelium, which is inhibited by IFN-γ (Robertson et al, 1992). Thus, IL-10, by inhibiting IFN-γ production of different cell types, is beneficial for maintaining GM-CSF secretion.

Furthermore, IL-3 production was reduced in CBA X DBA/2 mice and returned to normal when fetal resorption was prevented by oTP treatment. IL-3 and GM-CSF are known to have similar *in vitro* effect on trophoblast cells and *in vivo* effects on fetal growth. We demonstrated here that the production of IL-3 by fetoplacental unit correlates with fetal survival.

In summary, TH2 and TH1 cytokines have opposite effects on NK cell activity and CSF cytokine production. During normal pregnancy, TH2 cytokines are predominantly produced by the fetoplacental unit. This may be beneficial for the production of CSF cytokines, while inhibiting the production of TH1 cytokines and NK activity. On the other hand, the production of TH2 cytokines by the fetoplacental units of fetal resorption-prone mice is reduced, and the TH2/TH1 cytokine ratio is low. This could lead to the production of TH1 cytokines and the activation of NK cells, as well as the inhibition of CSF cytokine production, all of which would contribute to fetal demise.

Another interesting finding of our study is that oTP prevents fetal resorption. oTP is an antiluteolytic protein of 19 kd and is secreted by mononuclear cells of sheep trophectoderm (Bazer et al, 1989). oTP has a high amino acid sequence homology with interferons of the αII class (Imakawa et al, 1987). oTP is the only antiluteolytic protein secreted by sheep conceptus, ensuring the maintenance of progesterone secretion by the corpus leteum.

oTP is also immunosuppressive. oTP inhibits the proliferation of lymphocytes in response to PHA and IL-2 (Newton et al, 1989; Niwano et al, 1989). oTP thus is considered as a candidate for local inhibition of DTH or inflammatory phenomena during the peri-implantation period (Fillion et al, 1991).

Our investigation showed that oTP treatment led to increased production of IL-3, IL-4, and IL-10 at the maternal-fetal interface. This could be duo to the selective suppressive effect of oTP on the production of TH1 cytokines or NK cells at the fetoplacental unit, which in turn enhances the production of TH2 cytokines. As a consequence, the reduced ratios of TH2/TH1 cytokines at the fetoplacental units of fetal resorption-prone CBA X DBA/2 mice are reversed, and the fetal survival rate increased. In order to prove this, we must further investigate the *in vitro* effects of oTP on cytokine production in different cell types.

## D. General Discussion

In this thesis we have demonstrated that TH2-type cytokines, including IL-4, IL-5, IL-10, along with IL-3 and IFN-γ, are constitutively produced by the fetoplacental units during normal pregnancy. IL-3, IL-4, and IL-10 are produced by non-lymphoid cells at the maternal-fetal interface. IL-10 appears to be secreted by maternal decidual cells. The secretion of IL-3, IL-4 and IL-10 by cultured fetoplacental units correlates with fetal survival. This study expands our knowledge of the cytokine network at the maternal-fetal interface.

A major conclusion of the current study is that the production of TH2 cytokines at the maternal-fetal interface is an important parameter which may determine the overall status of the fetus and the maternal immune system (Figure 6.1).

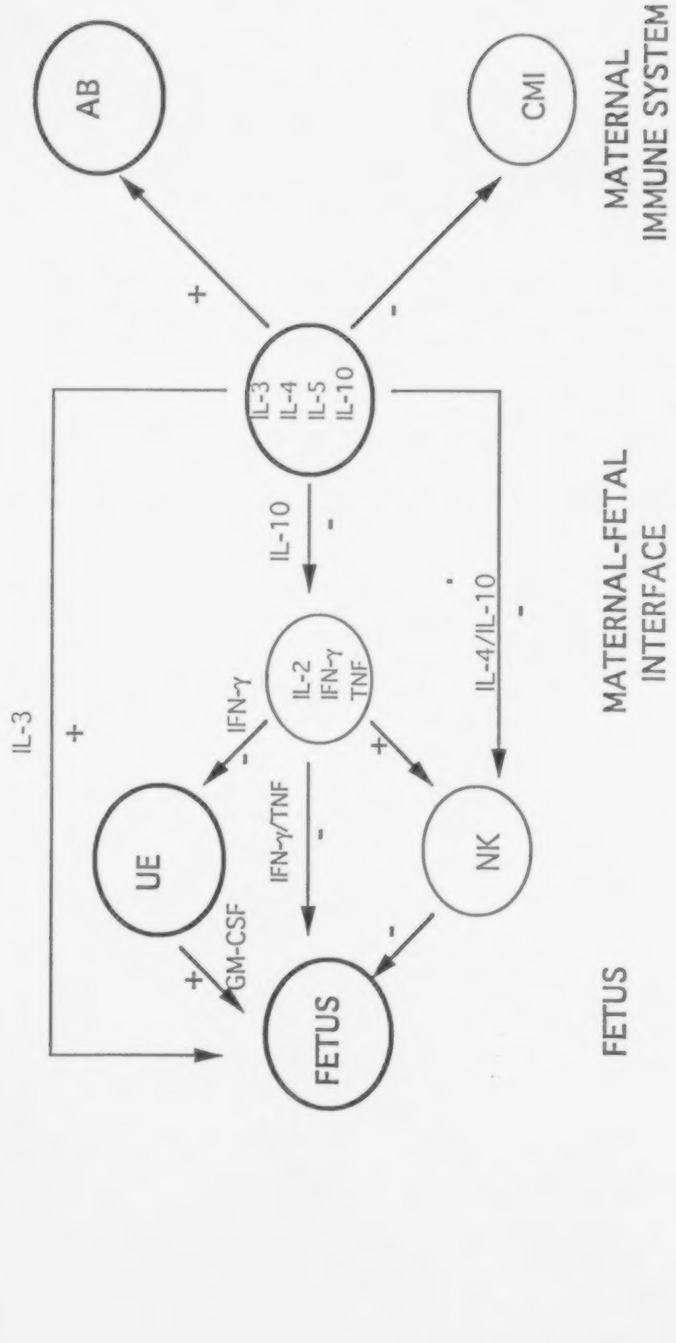
During normal pregnancy TH2 cytokines are predominantly produced, and the ratio of TH2/TH1 cytokines is high. This not only inhibits TH1 cytokine production and NK activity at the interface, but could also serve to maintain normal local production of CSF cytokines such as IL-3 and GM-CSF, which in turn would lead to normal fetal growth. In addition, it may well channel the maternal immune response towards antibody production and away from a cell-mediated response, diminishing maternal resistance to infections byintracellular pathogens as well as exacerbating certain autoimmune diseases and ameliorating others.

On the other hand, when significant amounts of TH2 cytokines are not produced and the TH2/TH1 cytokine ratio is lower than normal, the elevated concentration of TH1 cytokines IFN-γ and TNF may directly damage trophoblast cells. The TH1 cytokines could also activate NK cells and inhibit the production of CSF cytokines. This can lead to intrauterine fetal growth retardation as well as a restoration of the maternal DTH response.

This hypothesis needs to be further tested. Of the cytokines produced at the maternal-fetal interface, only GM-CSF and IL-3 have been shown to improve fetal survival. Since the production of IL-4 and IL-10 by the fetoplacental unit inversely correlates with thefetal

Figure 6.1 Hypothesized model of regulatory effects of TH2-like cytokines at the maternal-fetal interface on the fetus and the maternal immune system.

Detail see text in general discussion. +: upregulate; -: down-regulate; big circle: active stage; small circle: inactive or suppressed stage.


MATERNAL IMMUNE SYSTEM
MATERNAL-FETAL INTERFACE
FETUS
AB
CMI
+
-
IL-3 IL-4 IL-5 IL-10
IL-10
-
IL-4/IL-10
-
IL-3
+
IL-2 IFN-γ TNF
IFN-γ
-
IFN-γ/TNF
-
+
UE
NK
GM-CSF
+
-
FETUS

resorption rate in CBA X DBA/2 mice, further study of the *in vivo* effects of these two cytokines is needed in order to examine whether their production at the maternal-fetal interface is essential for maintaining a successful pregnancy.

We have began to examine the *in vivo* effect of IL-10 upon fetal survival. Since large amounts of pure mouse IL-10 were not available, we used the supernatant of BW5147 cells that had been transfected with a plasmid (pJFE14) containing an IL-10 cDNA fragment (kindly provided by Dr. J. F. Elliott, University of Alberta). The transfected cells were cultured in serum free medium. The supernatant was then collected, concentrated, and the amount of IL-10 in the supernatants was determined by ELISA. This supernatant was injected into CBA X DBA/2 mice, and fetal resorption rates were examined in Dr. Chaouat's laboratory. The fetal resorption rates of the mice that were injected with IL-10 were 10-20%, whereas the mice that were injected with PBS control had resorption rates of 70-80%. Because the IL-10 used in this experiment was not pure, it is difficult to exclude the possible contribution of other unknown proteins in the supernatant. Therefore, this data is not presented in this thesis. In order to draw a conclusion of whether IL-10 can prevent fetal resorption, more experiments need to be done using purer IL-10 with and without neutralizing antibody against IL-10. Preliminary experiments with an anti-IL-10 column indicated that the protection observed is due to IL-10. Antibody against IL-10 can also be injected into normal pregnant mice to examine whether IL-10 is essential for maintaining a successful pregnancy. Again, similar experiments can also be done with IL-4.

Another area that needs further investigation is the origin of cytokines produced at the maternal-fetal interface. *In situ* hybridization can be used to localize the cells that secrete IL-3, IL-4, IL-5, and IFN-γ. That will help to determine the different cellular sources of these cytokines and allow us to understand the complexity of the cytokine network at the interface.

The hypothesis presented here suggests that the maternal immune response is shifted away from TH1-mediated response and towards humoral responsiveness during pregnancy. This change compromises maternal cellular immunity, which plays the major defensive role against intracellular pathogens, such as tuberculosis and leprosy. On the other hand, when the maternal immune response is not biased away from cell-mediated immunity, e.g. if it is locked in a strong TH1 state by parasitic infection, infertility or fetal resorption may be the consequence.

This hypothesis is based on our work in mice. It is necessary to determine whether this hypothesis also applies to humans. One also needs to determine the cytokine profiles of patients who are infertile or are experiencing recurrent abortion. The correlation of cytokine production in this situation as well as in a variety of infectious diseases may provide insight into the immunological mystery of pregnancy. In addition, if TH2 cytokines are predominantly produced during human pregnancy, this information may help in the design of more rational strategies for therapy and prevention of infectious and autoimmune diseases. It may also help in the development of effective contraceptive methods as well as treatments for infertility and abortion.

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